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VARICELLA VIRUS–MONONUCLEAR CELL INTERACTION

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Varicella zoster virus (VZV) causes varicella (chickenpox), becomes latent in cranial nerve, dorsal root, and autonomic ganglia; and reactivates decades later to produce zoster (shingles). The main complication of zoster is postherpetic neuralgia (PHN), pain that persists for months and often years after zoster. VZV also causes chronic radicular pain without rash (zoster sine herpete). Viremia is associated with each stage of VZV infection. Viral DNA has been found in peripheral blood mononuclear cells (MNCs) of patients with varicella, zoster, PHN, and zoster sine herpete. In varicella, viremia contributes to the widespread distribution of skin lesions and infection of multiple organs. Although the role of viremia in other VZV-associated diseases is not as clear, the detection of VZV DNA (and sometimes VZV RNA and proteins) helps diagnose neurological diseases produced by VZV, has indicated that PHN may reflect a chronic VZV ganglionitis, and has established that VZV reactivates subclinically, especially in immunocompromised humans. *In vitro* studies have established that VZV can productively infect MNCs for a short time and have identified the subpopulations of MNCs that are infected. Finally, simian varicella virus (SVV) infection of nonhuman primates shares clinical, pathological, and virologic features with VZV in humans. Like VZV, SV viremia in nonhuman primates during acute infection plays an important role in the pathogenesis of SVV. Infectious virus can be isolated from MNCs, and SVV DNA can be detected in

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MNCs during varicella. Further, SVV DNA can be detected for months in MNCs of monkeys after experimental infection with SVV. Herein, we review the current literature related to VZV infection of MNCs during naturally occurring varicella, PHN, and zoster sine herpete in humans, including studies of experimental infection of human MNCs with VZV. We also review SVV MNC interaction during naturally occurring simian varicella and after experimental infection of primates with SVV.

I. Introduction

Varicella zoster virus (VZV) is an exclusively human alphaherpesvirus that causes varicella (chickenpox) and zoster (shingles) in humans. Varicella is a highly contagious, childhood exanthematous illness characterized by fever and a vesicular skin rash with cell-associated viremia. In the United States, 90% of new cases of varicella occur in individuals 1–14 years old, and approximately 95% of adults are seropositive. After primary infection, VZV establishes a lifelong latent infection in cranial nerve, dorsal root, and autonomic ganglia (Gilden *et al.*, 1983, 2001; Hyman *et al.*, 1983). Reactivation of latent VZV causes herpes zoster, most often in elderly or immunocompromised individuals. Zoster is characterized by pain and vesicular eruption generally restricted to one to three dermatomes at any level of the neuraxis (Head and Campbell, 1900). Persistence of pain 4–6 weeks after zoster is known as postherpetic neuralgia (PHN), and radicular pain without zoster rash is referred to as zoster sine herpete.

Viremia is associated with each stage of VZV infection. The typical incubation period for chickenpox is 14–15 days. In patients with varicella, viremia has been detected 4–6 days after exposure as well as more than a week before rash until a week after rash. Such prolonged viremia may actually reflect a biphasic course of virus entering blood, with the major secondary phase occurring after presumed viral replication in lymphoid tissue. Viremia during varicella contributes to the widespread distribution of skin lesions and infection of multiple organs. Although VZ viremia can also be found during zoster, it does not appear to be as important as in varicella. Viremia has also been documented in nonhuman primates during acute infection with simian varicella virus (SVV), which shares clinical (Padovan and Cantrell, 1986), pathological (Dueland *et al.*, 1992), and virologic (Clarke *et al.*, 1992; Gray *et al.*, 1992) features with VZV infection. SVV causes varicella in monkeys (Padovan and Cantrell, 1986; Soike *et al.*, 1984) and, like VZV, becomes latent in ganglia of the infected host at multiple

levels of the neuraxis (Mahalingam *et al*., 1991). SVV can be recovered during the incubation period and after the onset of varicella from naturally infected (Clarkson *et al*., 1967; Wolf *et al*., 1974) and experimentally infected monkeys (Dueland *et al*., 1992; Mahalingam *et al*., 2002; Soike *et al*., 1981a, 1982b; Wenner *et al*., 1977).

VZV DNA has been detected in blood mononuclear cells (MNCs) of varicella, zoster, PHN, and zoster sine herpete patients. Such detection has contributed greatly not only to diagnosis, but also in establishing VZV as the cause of multiple serious neurological disorders. Here, we review the current literature related to VZV infection of peripheral blood MNCs during varicella in humans and primates. We have highlighted the potential role and usefulness of finding VZV in patients with PHN and zoster sine herpete. We also discuss viremia in immunocompromised humans who have reactivated VZV without clinical symptoms or signs (so-called subclinical reactivation) and the use of a model of SVV infection in primates to study subclinical reactivation by the analysis of blood MNCs. Finally, we discuss experimental *in vitro* infection of MNCs, which has helped establish that productive infection of MNCs by VZV does occur.

II. Varicella

Primary VZV infection is established when infectious virus in respiratory droplets or vesicular fluid contacts mucosa of the upper respiratory tract (reviewed by Arvin, 1996). Initial viral replication is thought to occur in regional lymph nodes, followed by dissemination of virus into peripheral blood MNCs and transport of virus to liver and spleen where a second round of replication occurs. Skin epithelial cells, respiratory mucosa, and ganglia probably become infected during a secondary viremia, which continues after skin lesions appear.

VZ viremia has been difficult to study due to the low abundance of VZV-infected MNCs during varicella, zoster, and PHN and due to the cell-associated nature of the virus. However, VZV has been recovered from MNCs of varicella patients (Asano *et al*., 1985, 1990; Feldman *et al*., 1976; Ozaki *et al*., 1986), and VZV-specific DNA (Gilden *et al*., 1988; Koropchak *et al*., 1989; Mainka *et al*., 1998; Ozaki *et al*., 1994a, 1994b), transcripts (Mainka *et al*., 1998), and antigen (Mainka *et al*., 1998; Vafai *et al*., 1988) have been detected in MNCs during varicella. Data for each study are discussed below and summarized in Table I.

TABLE I
ISOLATION OF VZV AND DETECTION OF VIRAL DNA, RNA, AND PROTEIN IN BLOOD MNCS FROM VARICELLA PATIENTS

Days of sampling after onset of varicella	Virus isolation	DNA	RNA	Protein	Reference
+2 to +5[a]	Yes	nd[b]	nd	nd	Feldman and Epp (1976)
−8 to +18[a]	Yes	nd	nd	nd	Feldman *et al*. (1977)
−5 to −1	Yes	nd	nd	nd	Asano *et al*. (1985); Ozaki *et al*. (1986)
0 to +197	Yes[c]	Yes	nd	nd	Vonsover *et al*. (1987)
+2 to +56	nd	Yes	nd	nd	Gilden *et al*. (1988)
+2	nd	nd	nd	Yes	Vafai *et al*. (1988)
+1 to +2	nd	Yes	nd	nd	Koropchak *et al*. (1989)
+1 to +7	nd	Yes	nd	nd	Sawyer *et al*. (1992)
−14 to +14	nd	Yes	nd	nd	Ozaki *et al*. (1994a)
+1 to +8	nd	Yes	Yes[d]	Yes[e]	Mainka *et al*. (1998)

[a] Immunosuppressed cancer patients.
[b] Not done.
[c] Day +1.
[d] Days +1 to +5.
[e] Days +2 and +3.

A. Isolation of Varicella Zoster Virus from Mononuclear Cells

1. Immunosuppressed and Immunocompetent Varicella Patients

Infectious VZV was initially recovered from MNCs of four children with acute leukemia and varicella from 8 days before varicella to 18 days after rash (Feldman *et al*., 1976, 1977). VZV was later isolated from MNCs 11 days before varicella until 7 days after rash (Asano *et al*., 1985, 1990; Feldman *et al*., 1976, 1977; Ozaki *et al*., 1986).

In situ hybridization revealed VZV in MNCs from 24 to 48 h (Koropchak *et al*., 1989) and up to 56 days after varicella (Gilden *et al*., 1988), whereas dot-blot hybridization detected VZV DNA at multiple intervals in MNCs up to 197 days after varicella (Vonsover *et al*., 1987). Polymerase chain reaction (PCR) has detected VZV DNA in MNCs 1–7 days (Sawyer *et al*., 1992) and 1–8 days (Mainka *et al*., 1998) after varicella, and from 14 days before to 14 days after varicella (Ozaki *et al*., 1994a). VZV was not detected in varicella patients treated with acyclovir who were tested on the fourth day after

rash (Mainka *et al*., 1998). Ozaki and colleagues (1994a) detected VZV DNA in MNCs of one varicella patient 13 days before rash and 3 and 14 days after rash. In another patient, VZV DNA was detected in MNCs 11 days before rash and again at 14 days after varicella. VZV in blood MNCs early in the incubation period during rash and late in the incubation period that continues for days after rash is believed to reflect two distinct periods of viremia (Arvin, 1996; Ozaki *et al*., 1994a). Detection of VZV DNA in MNCs from 6 of 12 healthy children 28 days after vaccination with the Oka strain of VZV indicates a viremic phase in vaccinees and possible replication in some organs (Ozaki *et al*., 1994b).

In addition to DNA, VZV transcripts and antigen have been detected in MNCs during acute varicella. VZV immediate early (IE) and late (L) transcripts were detected by isothermal transcription-based nucleic acid amplification in MNCs up to 5 days after the onset of varicella (Mainka *et al*., 1998). Two L VZV glycoproteins (gl and gE) were detected by immunoprecipitation in MNCs 2 days after varicella (Vafai *et al*., 1988), and the VZV L glycoprotein E (gE) was detected by immunofluorescence in MNCs 2–3 days after rash (Mainka *et al*., 1998).

2. VZV in Subpopulations of MNCs

VZV has been detected in all subpopulations of MNCs, including T and B cells, monocytes, and macrophages. Ozaki *et al*. (1986) isolated VZV from total MNCs and lymphocytes on days 0 and 1 after rash and from nonadherent mononuclear cells (T and B lymphocytes), but not adherent cells (macrophages) 5 days before varicella. A later report described isolation of VZV primarily from nonadherent cells and T and B lymphocytes of 21 healthy children 3 days before rash (Asano *et al*., 1990). Together, data indicate that T and B cells, monocytes, and possibly macrophages are productively infected during the incubation period and during varicella. Analysis of VZV DNA in lymphocyte subpopulations by real-time quantitative PCR revealed the presence of equal amounts of VZV DNA in $CD4^+$ and $CD8^+$ T cells, and in B-cell and monocyte/macrophage fractions during the acute phase of varicella (Ito *et al*., 2001).

Using double staining with antibodies directed against VZV-gE and CD4/CD8 cells, respectively, Mainka *et al*. (1998) showed that 0.01 to 0.001% of these VZV-gE-expressing MNCs were infected. Two-thirds of these VZV-infected cells were T lymphocytes, and the remaining third were B lymphocytes and monocytes.

III. Zoster

After resolution of acute varicella and establishment of latency, VZV can reactivate from cranial nerve and dorsal root ganglia to cause zoster. Like varicella, VZV can be found in MNCs during zoster, but the role of viremia in the development of zoster and complications arising from zoster is unclear. Table II summarizes the isolation of VZV and detection of viral DNA, RNA, and protein in blood MNCs from zoster patients.

Infectious VZV was recovered from blood MNCs of immunosuppressed cancer patients 2–6 days after zoster (Feldman *et al*., 1977). In most of these patients, zoster was disseminated, presumably secondary to VZ viremia. To our knowledge, there has been only one report of isolation of infectious VZV from the blood of an immunocompetent patient with zoster affecting the maxillary distribution of the trigeminal nerve (Sato *et al*., 1979). A more recent attempt to isolate infectious virus from blood MNCs of immunocompetent zoster patients failed (Vonsover *et al*., 1987), perhaps due to the cell-associated nature of the virus and low levels of viral expression.

Most evidence of VZ viremia during zoster comes from the detection of VZV-specific DNA, RNA, or antigen in MNCs. VZV DNA was detected in MNCs 1–23 days after zoster by Southern and dot-blot hybridization (Gilden *et al*., 1987) and in ~1/100,000 MNCs up to 38 days after zoster rash by *in situ* hybridization (Gilden *et al*., 1988). Using nested PCR, Mainka *et al*. (1998) detected VZV DNA in MNCs from 11 of 71 zoster patients 1–6 days after rash. In addition, VZV

TABLE II
Isolation of VZV and Detection of Viral DNA, RNA, and Protein in Blood MNCs from Zoster Patients

Time of sampling after onset of zoster	Virus isolation	DNA	RNA	Protein	Reference
2 to 6 days[a]	Yes	nd[b]	nd	nd	Feldman *et al*. (1977)
Unknown	Yes	nd	nd	nd	Sato *et al*. (1979)
1 to 23 days	nd	Yes	nd	nd	Gilden *et al*. (1987)
0 to 37 days	nd	Yes	nd	nd	Gilden *et al*. (1988)
14 to 21 days	nd	nd	nd	Yes	Vafai *et al*. (1988)
1 to 6 days	nd	Yes	Yes	nd	Mainka *et al*. (1998)

[a] Immunosuppressed cancer patients.
[b] Not done.

IE and L transcripts were detected in MNCs from 9 of the VZV DNA-positive zoster patients 1–5 days after rash. Immunoprecipitation demonstrated the presence of the VZV L glycoproteins gpI and gpIV in MNCs from a zoster patient 2 weeks after rash (Vafai *et al*., 1988). Detection of VZV DNA, transcripts, and antigens in MNCs from zoster patients suggests that these cells are productively infected, although infection appears to be short lived.

IV. Postherpetic Neuralgia

The pain of zoster usually lasts 4–6 weeks, but pain that persists longer is called PHN. Although the mechanism of PHN is unknown, VZV-specific DNA (Devlin *et al*., 1992; Mahalingam *et al*., 1995; Vafai *et al*., 1988) and L glycoproteins (Vafai *et al*., 1988) have been detected in MNCs of PHN patients 1–8 years after zoster. In contrast, VZV DNA has been detected only up to 38 days after zoster in patients who did not develop PHN and not at all after the disappearance of zoster pain (Gilden *et al*., 1988). A later study that included zoster patients with and without PHN reported detection of VZV DNA by PCR in MNCs up to 8 years after zoster in 11/51 patients with PHN, but not in MNCs of 19 zoster patients without PHN who were analyzed 1–31 years after zoster or in any of 11 elderly age- and gender-matched subjects with no history of zoster (Mahalingam *et al*., 1995). Based on cumulative data (Gilden *et al*., 1988; Mahalingam *et al*., 1995; Vafai *et al*., 1988), we proposed that the detection of VZV DNA in MNCs of PHN patients may reflect the trafficking of MNCs through productively infected ganglia where viral persistence accounts for the continuous pain. While both Mainka *et al*. (1998) and Schunemann *et al*. (1999) have reported the absence of VZV DNA or RNA in MNCs of 16 PHN patients, we have studied a large number of patients over a period of many years. In one of our PHN patients, repeated analysis for more than a decade has revealed VZV DNA in MNCs on multiple occasions (unpublished data). Table III summarizes the isolation of VZV and detection of viral DNA, RNA, and protein in blood MNCs from PHN patients.

V. Zoster Sine Herpete

Nearly 100 years ago, Widal (1907) and Weber (1916) proposed the entity of zoster sine herpete, characterized as chronic radicular pain in a restricted dermatome distribution without rash. Further support

TABLE III
ISOLATION OF VZV AND DETECTION OF VIRAL DNA, RNA, AND PROTEIN IN BLOOD MNCS OF PATIENTS WITH POSTHERPETIC NEURALGIA

Time of sampling after onset of zoster	Virus isolation	DNA	RNA	Protein	Reference
1 to 4 years	nd[a]	Yes[b]	nd	Yes	Vafai *et al*. (1988)
Unknown	No	Yes	nd	nd	Devlin *et al*. (1992)
1 to 8 years	nd	Yes	nd	nd	Mahalingam *et al*. (1995)
2 months to 7 years	nd	No	No	nd	Mainka *et al*. (1998); Schunemann *et al*. (1999)

[a] Not done.
[b] DNA detected 1 year after zoster.

for the nosologic entity of zoster sine herpete was provided by Lewis (1958) in his description of multiple zoster patients who developed pain without rash in a dermatome different from the original site of zoster. The first virological evidence of zoster sine herpete came from a physician with acute trigeminal distribution pain that was followed by a four-fold rise in complement-fixing antibodies to VZV (Easton, 1970). Ultimate proof for the existence of zoster sine herpete came from the detection of VZV DNA, but not HSV DNA, in blood MNCs and cerebrospinal fluid (CSF) of one patient and in the CSF of a second patient with prolonged radicular pain; both were cured of pain after treatment with intravenous acyclovir (Gilden *et al*., 1994). We also encountered another patient with a 10-year history of thoracic radiculopathy and slowly progressive axonal sensory and motor neuropathy in the absence of rash whose CSF contained VZV antibody and MNCs contained VZV DNA (Amlie-Lefond *et al*., 1996). Terada *et al*. (1998) also found VZV DNA in blood MNCs of patients with zoster sine herpete.

In a larger study, McKendrick *et al*. (1999) detected VZV DNA in MNCs from 7/57 patients who presented with acute unilateral pain involving the trunk, arm or head, and neck, but no rash. VZV DNA was also found in 10/81 control subjects. Two patients in that study eventually developed rash characteristic of zoster, but it was unknown if these patients had VZV DNA in their MNCs. The age of the subjects varied from 23 to 82 years and, unfortunately, patients with acute unilateral pain over age 60 were not compared to age-matched controls. Meanwhile, evidence of VZV-associated viremia in zoster sine herpete and PHN patients supports the notion that

continuous radicular pain may result from a chronic ganglionitis secondary to active viral replication (Gilden *et al*., 1994; Mahalingam *et al*., 1995; Vafai *et al*., 1988).

VI. Subclinical Reactivation

Subclinical reactivation of latent VZV was initially hypothesized by Hope-Simpson (1965) as a means of bolstering immunity. Subclinical reactivation was documented in immunocompromised patients by the enzyme-linked immunosorbent assay and assessment of the lymphocyte proliferation response to VZV antigen (Ljungman *et al*., 1986) and PCR (Wilson *et al*., 1992). Several studies have utilized sensitive PCR techniques to demonstrate subclinical VZV-associated viremia in VZV-seropositive immunocompetent healthy adults (Devlin *et al*., 1992; Gilden *et al*., 1992; Mainka *et al*., 1998; McKendrick *et al*., 1999). In two of these studies (Devlin *et al*., 1992; Gilden *et al*., 1992), a partial VZV genome was detected in 1/22 healthy adults under age 60 and in 4/16 elderly adults over age 60. Mainka *et al*. (1998) detected VZV DNA in MNCs of 5/149 healthy adults, but only after nested PCR indicated a low viral load. Blood samples obtained from the positive individuals weeks later were negative, indicating that MNCs were not latently infected with VZV. In the study by McKendrick *et al*. (1999), VZV was detected in MNCs of 10/81 control patients. Together, data support Hope-Simpson's hypothesis that VZV reactivates subclinically both in normal healthy adults and in immunocompromised individuals.

VII. Simian Varicella Virus Infection in Nonhuman Primates

Like VZV, SVV infection produces viremia, and infectious virus can be recovered from blood MNCs of naturally infected monkeys (Clarkson *et al*., 1967; Wolf *et al*., 1974) and from 2 to 11 days after experimental infection of nonhuman primates (Dueland *et al*., 1992; Iltis *et al*., 1982; Soike *et al*., 1981a, 1981b; Wenner *et al*., 1977). Evidence for reactivation in SVV-infected monkeys is limited, but has been observed in monkeys exposed to social and environmental stress (Soike *et al*., 1984). Reactivation often presents as a whole-body rash, and virus has been isolated from skin vesicles, but not from blood. Table IV summarizes the isolation of SVV and detection of viral DNA in blood MNCs from SVV-infected nonhuman primates.

TABLE IV
ISOLATION OF SVV AND DETECTION OF VIRAL DNA IN BLOOD MNCS FROM INFECTED NONHUMAN PRIMATES

Days of sampling after inoculation	Virus Isolation	DNA	Reference
Unknown[a]	Yes	nd[b]	Clarkson *et al*. (1967); Wolf *et al*. (1974)
2 to 8	Yes	nd	Wenner *et al*. (1977)
3 to 7	Yes	nd	Iltis *et al*. (1982)
3 to 11	Yes	nd	Dueland *et al*. (1992); Soike *et al*. (1981a,b)
5 to 59	Yes[c]	Yes	Mahalingam *et al*. (1995)
10[a]	No	Yes	Mahalingam *et al*. (2002)
6 to 700	Yes[d]	Yes	White *et al*. (2002b)

[a] Naturally infected monkeys.
[b] Not done.
[c] Virus isolated on days 6 to 10 after inoculation.
[d] Virus isolated 6 days after inoculation, but not 14 months later.

In three separate studies from our laboratory (Mahalingam *et al*., 2001, 2002; White *et al*., 2002b), SVV DNA was detected in MNCs of monkeys experimentally infected with SVV. Using PCR, Mahalingam *et al*. (2001, 2002) detected SVV DNA in MNCs 5–59 days after intratracheal inoculation of monkeys with SVV and in one SVV-seronegative monkey 10 days after exposure to a monkey that had been inoculated intratracheally with SVV. White *et al*. (2002b) detected SVV DNA in MNCs from monkeys with acute varicella at 7 and 10 days after infection, in one group of intratracheally infected monkeys at 10 and 12 months after infection, and in another group of intratracheally infected monkeys for 11–23 months. PCR analysis of MNC subpopulations during acute viremia (10 days after infection) revealed the presence of SVV DNA in $CD4^+$ and $CD8^+$ T cells, $CD20^+$ B lymphocytes, and in the $CD14^+$ monocyte/macrophage fraction (White *et al*., 2002b). These findings complement work by Ito *et al*. (2001), who showed that VZV DNA is present in all MNC subpopulations during acute varicella in humans. Analysis of MNC subpopulations in our laboratory at 14 and 23 months after experimental infection revealed SVV DNA in $CD4^+$ and $CD8^+$ cells, but not in $CD14^+$ or $CD20^+$ cells. Infectious SVV was recovered from monkeys during acute varicella, but not 14 months after infection. Thus, SVV appears to infect all MNC subpopulations during acute varicella, but after resolution of the primary infection, virus is cleared from B cells and remains in T cells.

It is not yet clear whether the detection of SVV DNA in $CD4^+$ and $CD8^+$ MNCs many months after the resolution of acute varicella reflects continued infection of cells that began at the time of acute varicella or represents infection acquired by MNCs trafficking through productively infected tissues. SVV-specific DNA and transcripts representing all herpesvirus gene classes have been detected in ganglia and viscera (White *et al*., 2002a) of the same monkeys whose blood MNCs were positive for SVV DNA 10 months after infection (White *et al*., 2002b). Detection of SVV DNA in blood MNCs in addition to abundant viral transcription suggests infection of MNCs after trafficking through productively infected ganglia and viscera. In light of the consistent detection of SVV DNA in MNCs for over a year in multiple monkeys examined 10–23 months after experimental infection (White *et al*., 2002a) without any clinical signs of reactivation, it seems unlikely that episodic subclinical reactivation accounts for these findings. Instead, blood MNCs are likely infected with SVV after trafficking through productively infected tissue of these chronically infected monkeys. Although the monkeys did not appear to be in pain, persistence of SVV DNA in blood MNCs from SVV-infected monkeys may correspond to VZ viremia in PHN patients where viral DNA has been detected for years after zoster or during zoster sine herpete (Gilden *et al*., 1992; Mahalingam *et al*., 1995; Vafai *et al*., 1988). Further analysis of SV viremia in the nonhuman primate model of varicella will likely continue to enhance the understanding of human varicella and the relationship of viremia to virus in tissue.

VIII. Infection of Mononuclear Cells *in Vitro*

The presence of VZV in MNCs at different stages of VZV infection has been well documented. However, it is still controversial which MNC subpopulations harbor VZV at various stages and which cells, if any, are productively infected . Infectious VZV has been recovered from both nonadherent MNCs (lymphocytes) (Asano *et al*., 1990; Ozaki *et al*., 1986) and adherent MNCs (macrophages) (Asano *et al*., 1990) during acute varicella. Mainka *et al*. (1998) detected VZV antigen primarily in T cells, but also in B cells and monocytes from varicella patients.

Results of *in vitro* studies of VZV infection of MNCs have been conflicting. Table V summarizes the isolation of VZV and detection of viral DNA, RNA, and protein after *in vitro* infection of MNCs. In one study, VZV infection of fresh MNCs yielded few infected cells, but infectious virus and antigen were detected after VZV infection of cultured

TABLE V
ISOLATION OF VZV AND DETECTION OF VIRAL DNA, RNA, AND PROTEIN AFTER *IN VITRO* INFECTION OF MNCS

Mononuclear cell type	Virus isolation	DNA	RNA	Protein	Reference
Monocyte-derived macrophage	Yes	nd[a]	nd	Yes	Arbeit *et al*. (1982)
Cultured mononuclear cells from healthy adults	nd	Yes[b]	nd	Yes	Gilden *et al*. (1987); Ku *et al*. (2002)
EBV-transformed B cells	Yes	nd	nd	Yes	Koropchak *et al*. (1989)
Mitogen-stimulated T cells	Yes	nd	nd	Yes	Koropchak *et al*. (1989); Ku *et al*. (2002)
Mitogen-stimulated B cells	No	nd	nd	No	Koropchak *et al*. (1989)
$CD4^+$ T-cell hybridoma cell line	No	nd	nd	Yes	Zerboni *et al*. (2000)
Cultured human umbilical cord blood MNCs	Yes	nd	nd	Yes[c]	Soong *et al*. (2000)
Cultured human tonsil cells	nd	nd	nd	Yes[d]	Ku *et al*. (2002)

[a] Not done.
[b] Detected in T cells, B cells, monocytes, and macrophages.
[c] Detected primarily in $CD3^+$ T cells.
[d] Detected primarily in $CD4^+$ T cells.

macrophage cells derived from monocytes (Arbeit *et al*., 1982). We detected VZV antigens in cultured MNCs by immunoprecipitation and immunofluorescence 3 and 7 days after infection, respectively (Gilden *et al*., 1987). In the same study, VZV-infected MNCs sorted using flow cytometry revealed VZV DNA primarily in B cells, but also in T cells and monocyte/macrophage cells. However, a lack of VZV-specific [^{3}H]thymidine incorporation in VZV-infected cultured MNCs indicated the absence of viral replication. Using cultured human umbilical cord blood MNCs infected with VZV, Soong *et al*. (2000) showed that 4% of cells were infected, mostly $CD3^+$ T cells. The VZV-infected umbilical cord MNCs expressed VZV antigens and released infectious virus, indicating productive infection.

VZV replication, antigens, and infectious virus have been detected in Epstein–Barr virus-transformed B lymphocytes and in mitogen-stimulated T cells, but not in mitogen-stimulated B cells (Koropchak *et al*., 1989). Despite the presence of VZV DNA in more than 75% of the cells, viral replication was demonstrated in only a small percentage of the activated T cells, indicating an abortive infection in most of the cultured T cells. The investigators suggested that activated T cells,

present during the host response to primary varicella infection, might potentiate viremia. VZV infection of a $CD4^+$ T-cell hybridoma cell line yielded VZV immediate-early, early, and late proteins in 30% of cells, but no infectious progeny (Zerboni *et al*., 2000).

Ku *et al*. (2002) recently reported *in vitro* VZV infection of cells from human tonsil tissue. Using anti-VZV IgG staining and flow cytometry, these investigators showed that VZV can productively infect $CD4^+$ T cells, especially those exposed to mitogen, as shown previously by Koropchak *et al*. (1989). Moreover, electron microscopic analysis demonstrated VZ virions in infected cells (Ku *et al*., 2002). Based on the marked susceptibility of T cells to VZV infection, the authors suggested that these cells may be important targets during primary upper respiratory infection by VZV.

T-cell tropism of VZV has also been demonstrated in a SCID-hu mouse carrying human fetal thymus and liver implants (Moffat *et al*., 1995). Infectious VZV was recovered from human lymphocytes circulating in the mice until day 21 after infection, and failure to recover infectious virus correlated with a decline in $CD4^+$ and $CD8^+$ cells. Fluorescence-activated cell-sorting analysis of VZV-infected human T cells from the thymus/liver implants revealed viral proteins in $CD4^+$ and $CD8^+$ cells.

In vitro and SCID-hu mouse data suggest that most subpopulations of MNCs can take up VZV DNA and that viral proteins can be expressed, but the infection is usually abortive. Because infectious virus can be recovered from MNCs during primary varicella, clearly there are cells in this population that support productive infection. To date, most of the evidence points to T cells as the subpopulation in which VZV most readily replicates and produces viral progeny.

IX. Concluding Remarks

VZV-associated viremia and lymphotropism have been well documented at all stages of VZV infection, including primary varicella, zoster, PHN, zoster sine herpete, and even in normal healthy adults. During primary varicella, MNCs are productively infected, which most likely contributes to the spread of virus, and there is growing evidence that T cells facilitate VZV-associated viremia at this stage. Viremic evidence for the subclinical reactivation of VZV in normal healthy adults suggests the role of immunity to VZV throughout the life of the host, possibly helping to prevent symptomatic manifestations of VZV reactivation until later in life.

VZV-specific DNA, transcripts, and protein have been detected in MNCs of zoster and PHN patients, indicating productive infection. However, with one exception (Sato *et al*., 1979), attempts to recover infectious VZV from MNCs of immunocompetent zoster patients have failed (Gilden *et al*., 1988; Vonsover *et al*., 1987). Similar findings have been documented *in vitro* after infecting cultured MNCs with VZV (Gilden *et al*., 1987; Zerboni *et al*., 2000). In zoster and PHN patients, a lack of detectable infectious virus may rest in the cell-associated nature of the virus, in the low level or abortive infection of MNCs, or in the decreased frequency of MNC cell types that support productive infection during these stages compared to primary infection.

Despite a lack of recoverable virus from blood, detection of VZV DNA in MNCs of PHN and zoster sine herpete patients supports the notion that productive infection in ganglia contributes to these conditions. The ability to detect VZV DNA in MNCs of some of these patients is also a useful tool for diagnosis, leading to prompt treatment of the syndromes. Detection of SVV DNA and SVV-specific transcripts representing all herpesvirus gene classes in MNCs and ganglia, respectively, from the same monkey months after experimental infection further supports the previously-mentioned hypothesis. Viremic evidence for subclinical reactivation of VZV in normal healthy adults suggests that immunity to VZV is bolstered throughout life, helping to forestall clinical signs of VZV reactivation.

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ADVANCES IN VIRUS RESEARCH, VOL 62

EVOLUTION OF CELL RECOGNITION BY VIRUSES: A SOURCE OF BIOLOGICAL NOVELTY WITH MEDICAL IMPLICATIONS

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I. Introduction

The penetration of animal viruses into cells is the result of an active process of specific interactions with cell surface macromolecules. Differentiated organisms do not express identical sets of surface macromolecules on different cell types, tissues, and organs. This basic feature of organisms results in a compartmentalization of susceptibilities to infection by viruses. As observed repeatedly in nature, different viruses infect distinct target cells in one or a group of host species. This

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is the expected evolutionary outcome of a population equilibrium between organisms and their parasites that must have been favored by mutual long-term interactions. Although permissivity of virus entry into cells is by no means the only determinant of host cell tropism, it is certainly an important element with implications in viral pathogenesis. Changes in the specificity of virus entry into cells may be of consequence also for viral disease emergence and disease prevention and control.

Early reports (reviewed in McLaren *et al*., 1959) suggested the presence of receptors for bacteriophage in *Escherichia coli* cells and for influenza virus, Newcastle disease virus, and enteroviruses in some animal cells. The studies of McLaren, Holland, and their colleagues with poliovirus (Holland, 1961; Holland and McLaren, 1959; McLaren *et al*., 1959) indicated that the productive adsorption of poliovirus was associated with specific antigenic structures possessed only by susceptible primate cells (or subcellular cell debris). We term viral receptor any cell surface macromolecule involved in the recognition of the cell by a virus or in the penetration of a virus into the cell. For simplification, we do not establish a difference between receptor and coreceptor (Young, 2001) because such a distinction is at times ambiguous, and it is not essential to the main aims of the present review. However, we maintain the term coreceptor when referring to some of the molecules that participate in retrovirus entry, as in this case the term is amply employed in the literature. The problems addressed in this review are in the interphase between virus evolution and cell recognition by viruses. Topics include changes in receptor usage, shared use of the same receptors by different viruses, and coevolution of antigenicity and host cell tropism. We then review briefly some of the implications of flexibility in receptor usage for the host range of viruses, for the emergence of viral diseases, and consequences for the use of viruses as vectors for gene delivery. In this regard, we discuss the possible involvement of viruses as agents of lateral gene transfer during the evolution of cellular life on earth as a means to accelerate functional diversification of cells and cell collectivities. In none of theses topics can we (or intend to) be exhaustive. Selected examples are used to unveil the implications of the highly dynamic nature of viral genomes for modifications of receptor specificity, particularly for those viruses with RNA as genetic material. Recent introductions to the mechanisms of virus entry into cells can be found in general virology textbooks (Cann, 2001; Flint *et al*., 2000). Among excellent reviews on viral receptors are those published by Wimmer (1994), Weiss and Tailor (1995), Miller (1996), Hunter (1997), Evans and Almond (1998), Sommerfelt (1999), Berger *et al*. (1999), Nemerow (2000), Schneider-Schaulies (2000),

Skehel and Wiley (2000), Rossmann *et al*. (2000, 2002), Spear *et al*. (2000), Speck *et al*. (2000), Barton (2001), Dragic (2001), Young (2001), Eckert and Kim (2001), Shukla and Spear (2001), Goldsmith and Doms (2002), Hogle (2002), Kunz *et al*. (2002), and Bomsel and Alfsen (2003).

Virus evolution has been covered in Domingo *et al*. (1999, 2001), Crandall (1999), and Flint *et al*. (2000). A brief review on the evolution of cell tropism of viruses was published by Baranowski *et al*. (2001). The following sections introduce current concepts of virus evolution and how viral population dynamics affects changes in cell recognition.

II. Basic Concepts of Virus Evolution

A. Types of Interactions between Viruses and Cells

Viruses are highly diverse molecular parasites of cells that have an intracellular phase of replication and an extracellular stage in the form of discrete particles. Despite their remarkable diversity in size, shape, and biological properties, a few definitive features are common to all viruses: (i) they have DNA or RNA (but not both) as genetic material; (ii) their genome encodes a distinctive genetic program, and (iii) the expression of this program, which results in virus replication, is totally dependent on cell structures and cell metabolism.

In their dependence on cells, viruses can produce a wide range of perturbations, such as modifications of specialized (luxury) functions, without an immediate effect on cell survival, or they may cause cell death by apoptosis or necrosis. Viral infections may be inapparent or they may cause acute or chronic disease either directly by affecting cell subpopulations or indirectly by triggering immunopathological responses (Mims *et al*., 2001; Nathanson *et al*., 1997). These disparate effects on cells have not been correlated with genome type, virion structure, or receptor usage, presumably because many host functions, as much as viral functions, influence the outcome of an infection.

The genetic complexity of viruses, as reflected in the size of their genome, varies from a few thousand nucleotides in the case of RNA viruses (the genome of bacteriophage MS2 is 3569 nucleotides long, and that of the largest coronaviruses comprises about 32,000 nucleotides) to a broader range in the case of DNA viruses (the double-stranded DNA of hepatitis B virus has about 3200 bp, whereas the large poxviruses, iridoviruses, and herpesviruses have DNA of 130,000 up to 370,000 bp). Genome complexity appears to influence the strategies

of the interactions between viruses and cells. Hosts must have evolved defense mechanisms to limit virus replication at the expense of their cells, and in turn, viruses must have evolved mechanisms to counteract cell responses (at least to survive to be an object of interest for a review article). The most complex DNA viruses encode a number of proteins that may or may not have a cellular homologue and that may modulate host defense responses. Classical examples are the glycoprotein C of herpes simplex virus, which blocks complement activation, or proteins E3/19K and E1a of adenovirus, which suppress major histocompatibility complex (MHC) class I and class II molecules that are required for the T-cell recognition of infected cells. Other viral proteins are homologues of cytokines, chemokines, or their receptors or may induce or inhibit apoptosis. An increasing number of viral-coded, immunomodulating proteins is being discovered, some with seemingly redundant functions, and others with multiple effects on cells, playing active roles as determinants of virus survival and pathogenesis (reviews in Alcamí and Koszinowski, 2000; Alcamí, 2003; Seet *et al*., 2003; Xu *et al*., 2001). Host-interfering proteins may also be expressed by RNA viruses (HIV-1 nef, RNA silencing suppressor B2 in flock house virus, several interferon antagonists such as NS1 of human influenza virus A, etc.), albeit their number appears to be more limited than for complex DNA viruses. This probably reflects two fundamentally different viral strategies to cope with host defenses: interaction versus evasion.

In contrast to the complex DNA viruses, RNA viruses have condensed essential genetic information for replication in a minimal number of nucleotides. Compressing mechanisms include the presence of overlapping reading frames; ambisense RNA; common leader RNA sequences for the synthesis of subgenomic messenger RNAs; untranslated regions, which include signals for RNA replication and protein synthesis; *cis*-acting regulatory elements within open reading frames; RNA editing; partial read-through of termination codons, leading to two forms of a protein that differ in a carboxy-terminal extension; leaky ribosome scanning with initiation of protein synthesis occurring at two in-frame AUGs, leading to two forms of a protein that differ by an amino-terminal extension; ribosome hopping, shunting, and bypassing; ribosome frameshifting, resulting in a change of the order of triplet reading to yield a single fused polypeptide from two overlapping open reading frames; and synthesis of a polyprotein, which is then cleaved to produce functional intermediates and fully processed proteins, with processing intermediates and processed proteins having distinct functional roles in interaction with viral RNA, viral proteins, or cellular proteins. In addition, many viral proteins appear to be

multifunctional, thereby expanding the role of a single nucleotide stretch in the completion of a virus replication cycle (reviews in Flint *et al*., 2000; Knipe and Howley, 2001; Semler and Wimmer, 2002). Genome compactness imposes a conflict between the requirement of genetic variation to permit adaptation to changing environments and the need to maintain infectivity in genomes in which virtually every single nucleotide appears to be involved in some structural or functional role. Genetic variation to escape from selective constraints is generally reflected in the survival of subpopulations of genomes that may show little alteration in replication capacity (fitness) or, despite a reduction of fitness, may still replicate to generate new mutant distributions of higher fitness.

B. Genetic Variation and the Dynamics of Viral Populations

The tolerance of viral genomes (or other replicons) to accept mutations decreases with genome complexity (Domingo *et al*., 2001; Eigen and Biebricher, 1988). RNA viruses display mutation rates and frequencies in the range of 10^{-3} to 10^{-5} errors per nucleotide copied (Batschelet *et al*., 1976; Drake and Holland, 1999), values that imply the continuous generation of dynamic mutant distributions in a replicating RNA virus population. These high mutation rates in RNA genomes would be incompatible with maintenance of the genetic information contained in large viral or cellular DNA genomes (Eigen and Biebricher, 1988). This evolutionary adaptation of mutation rates is mirrored in the biochemical activities of the relevant DNA and RNA polymerases. Cellular DNA polymerases involved in DNA replication generally contain a $3' \rightarrow 5'$ exonuclease proofreading–repair activity capable of excising misincorporated nucleotides to allow incorporation of the correct complementary nucleotide prior to further elongation of the nascent DNA chain. Such a proofreading–repair activity is absent in viral RNA replicases and reverse transcriptases, as evidenced by both structural and biochemical studies (Menéndez-Arias, 2002; Steinhauer *et al*., 1992). Furthermore, a number of postreplicative repair pathways are active on double-stranded DNA but cannot act on RNA, therefore contributing to a final 10^5- to 10^6-fold higher average copying fidelity during cellular DNA replication than during RNA genome replication (Domingo *et al*., 2001; Drake and Holland, 1999; Friedberg *et al*., 1995; Goodman and Fygenson, 1998).

Viruses exploit the same mechanisms of genetic variation as cells: mutation, homologous and nonhomologous recombination, and genome segment reassortment in the case of viruses with segmented

genomes. Different virus families vary in the extent of utilization of these different variation mechanisms. For example, homologous recombination is very active in positive-strand RNA viruses and retroviruses, but appears to be rare in negative-strand RNA viruses. Because of general high mutation rates, RNA virus populations consist of complex mutant distributions termed viral quasispecies (Domingo *et al*., 2001; Eigen, 1996; Eigen and Biebricher, 1988; Eigen and Schuster, 1979; Nowak, 1992). Quasispecies was formulated as a general theory of molecular evolution by M. Eigen and colleagues to describe self-organization and adaptation of simple early replicons that could occur at early stages of the development of life (Eigen and Biebricher, 1988; Eigen and Schuster, 1979). Although the initial quasispecies theory involved mutant distributions in equilibrium, extensions of the theory to finite replicon populations subjected to environmental changes have been developed (Eigen, 2000; Wilke *et al*., 2001). Therefore, the quasispecies theory was instrumental in understanding the population structure and dynamics of RNA virus populations. Virologists use an extended definition of quasispecies to signify dynamic distributions of nonidentical but closely related mutant and recombinant viral genomes subjected to a continuous process of genetic variation, competition, and selection, and which act as a unit of selection (Domingo, 1999). It must be stressed that because of high mutation rates (Batschelet *et al*., 1976; Drake and Holland, 1999), each individual genome in a replicating population, which includes a number of distinctive mutations, has only a fleeting existence because new mutations are arising continuously, even in a single infected cell. Thus, an RNA virus genome population may be defined statistically but it is essentially indeterminate at the level of individual genomes (Domingo *et al*., 1978, 2001). Mutant swarms are subjected unavoidably to competition, and those mutant distributions best adapted to replicate in a given environment are those that dominate the population at a given time. Unfit mutant distributions are subjected to negative selection and kept at low (sometimes undetectable) levels in the population. Unfit mutant distributions in one environment may nevertheless be fit in a different environment, and a modulation of frequencies of genome subpopulations is the key to adaptability of RNA viruses. The biological relevance of the quasispecies nature of RNA viruses stems from the fact that mutant spectra may contain genomes with altered biological properties, including modified cell recognition capacity. Relevant parameters are the number of mutations per genome, genome length, and viral population size, as documented with several virus systems both in cell culture and *in vivo* (Table I).

TABLE I
BIOLOGICAL RELEVANCE OF QUASISPECIES DYNAMICS FOR RNA VIRUSES[a]

Relevant parameters
1. Average number of mutations per genome found in a viral population: typically it ranges from 1 to 100
2. Virus population size: very variable but can reach 10^9 to 10^{12} in some infections *in vivo*
3. Genome length: 3 to 33 kb, with compact genetic information
4. Mutations needed for a phenotypic change: one or few mutations, as documented amply in the text for changes in receptor recognition specificity
Examples of phenotypic changes in RNA viruses dependent on one or few mutations
1. Antigenic variation (antibody-, CTL-escape)
2. Virulence
3. Altered pattern of gene expression
4. Resistance to antiviral inhibitors
5. Cell tropism and host range (the topic of this review)

[a] Based on many published studies reviewed in Domingo *et al*. (1985, 1999, 2001), Crandall (1999), Flint *et al*. (2000), and DeFilippis and Villarreal (2001).

Despite the unlikely occurrence of generalized high mutation rates in complex DNA viruses, hot spots for variation at sequence repeats and extensive diversity among isolates of DNA viruses have been observed (Lua *et al*., 2002; Smith and Inglis, 1987). Furthermore, small DNA viruses, such as plant geminiviruses or animal parvoviruses, show features of quasispecies dynamics, similar to RNA viruses (Isnard *et al*., 1998; Lopez-Bueno *et al*., 2003; reviewed in Domingo *et al*., 2001).

Diversification of viruses within infected organisms, even when it involves limited genetic change, is of consequence for viral pathogenesis and persistence. This is because one or a few mutations may suffice to change important biological properties of viruses (such as host cell tropism, resistance to antibodies, to cytotoxic T lymphocytes or to inhibitors, among other traits; Table I). Small numbers of mutations in components of mutant spectra are easily attainable by diversifying populations of viruses during acute or chronic infections. Even when a mutation that confers a phenotypic change results in a modest fitness decrease, compensatory mutations can have an opportunity to rescue genomes with normal or nearly normal fitness values (Cassady *et al*., 2002; Escarmís *et al*., 1999, 2002; Lázaro *et al*., 2002; Liang *et al*., 1998; Nijhuis *et al*., 1999; Wang *et al*., 1996; Yuan and Shih, 2000).

Viral fitness determined *ex vivo* may be a relevent indicator of disease progression *in vivo* (Ball *et al.*, 2003). Within-host variation is the first step in the process of long-term diversification of viruses in successions of transmission events from infected to susceptible hosts. Comparison of consensus nucleotide sequences of independent isolates of the same virus originated from a single source of infection allows a calculation of the rate of evolution. As expected from the complex quasispecies dynamics, rates of evolution for RNA viruses are not constant with time (a "clock" does not operate) and are often in the range of 10^{-2} to 10^{-4} substitutions per nucleotide and year. These values can also vary for different genomic segments of the same virus. In sharp contrast, rates of evolution for cellular genes have been estimated in 10^{-8} to 10^{-9} substitutions per nucleotide and year. Interestingly, DNA viruses, even those with small genome size, display widely different rates of evolution. As an example, such rates have been estimated in 1.7×10^{-4} substitutions per nucleotide and year for canine parvovirus and in the range of 1×10^{-7} to 3×10^{-8} substitutions per nucleotide and year for some papovaviruses (reviewed in Domingo *et al.*, 2001).

Genomic consensus sequences of independent isolates of a virus allow the establishment of phylogenetic relationships by the application of classical procedures of population genetics (Doolittle, 1996; Page and Holmes, 1998; Weiller *et al.*, 1995). Such procedures have confirmed the extensive diversity of extant viruses; it has not been possible to derive a genetic tree that relates the known viruses, not even DNA or RNA viruses separately. This, together with the disparate replication strategies exhibited by viruses, suggests multiple origins for the viruses we study today: viruses are polyphyletic. For viruses that infect distantly related host species, such as the herpesviruses, a parallelism between host phylogeny and virus phylogeny has been observed (McGeoch and Davison, 1999). This suggests a host–virus cospeciation, implying deep evolutionary roots of viruses with their hosts (Gorbalenya, 1995; McGeoch and Davison, 1999) (discussed further in Section V,D).

While we have attained some understanding of the mechanisms involved in the generation of diversity, little is understood of the forces that favor the dominance of some virus types over others in nature. According to the International Committee on Taxonomy of Viruses (van Regenmortel *et al.*, 2000), there are about 3600 virus species recognized and more than 30,000 different viruses, strains, and subtypes. Considering that each "individual" RNA virus circulates as a dynamic quasispecies, the diversity of viral genomes on earth is astonishing.

Each replication-competent component of a quasispecies distribution can, in principle, initiate a virus diversification process to generate a spectrum of genotypes and phenotypes. RNA replicons constitute a highly dynamic "RNA world" in a relatively more static [but only relatively! (Bushman, 2002)] DNA-based biosphere of differentiated organisms, as noted two decades ago by Holland and colleagues (1982). The central objective of this review is to examine the effect of virus variation on cell recognition and some of its biological consequences.

III. Nature of Viral Receptors

Viruses recognize target cells by binding to specific receptor molecules at the cell surface. Until the mid-1980s, the only virus receptors that were identified unequivocally were sialic acids for the myxoviruses and paramyxoviruses (Haywood, 1994). Since that time, there has been a deluge of new data about the nature of cell surface molecules participating in viral entry. The molecules that have so far been characterized as viral receptors belong to different families of proteins, carbohydrates, and lipids, often organized as cell surface complexes. Receptors are normally involved in critical cellular functions such as signal transduction, cell adhesion, immune modulation, enzymatic activities, and for some receptors no cellular function has been identified yet (Table II). It is not clear why some macromolecules and not others act as viral receptors. It has been suggested that one reason is their abundance and availability on the cell surface combined with their capacity to bind and/or internalize viruses while triggering initiation of the virus replication cycle. By participating in the first step of virus infection, receptors divert from their usual activity in cellular metabolism and provide yet another example of molecular parasitism, essential in the life cycle of viruses.

It has not been possible to predict the type of receptors likely to be used by a virus from its phylogenetic position, nor from its biological properties. The diversity of receptors is reciprocated by their unforeseeable exploitation by viruses, independently of their genome structure and replication strategy. The picornaviruses, which share structural features in their capsids, may use as receptors molecules as diverse as integrins, glycoproteins of the immunoglobulin gene superfamily, decay accelerating factor (DAF or CD55), or sialic acid or sialylated proteins or lipids (Table II). A survey of the more complex coronaviruses does not modify the picture: receptors for coronaviruses include aminopeptidase N, the sialic acid *N*-acetyl-9-*O*-acetylneuraminic acid, *N*-glycolylneuraminic

TABLE II

SOME CELL SURFACE COMPONENTS PROPOSED TO BE INVOLVED IN VIRUS ENTRY INTO CELLS

Receptor class	Cellular structure[a]
Extracellular matrix components, sugar derivatives and lipids	Galactosylceramide
	Gangliosides
	Glycosaminoglycans (heparan and chondroitin sulfates)
	Phospholipids
	Sialic acid (*N*-acetylneuraminic acid, *N*-acetyl-9-*O*-acetylneuraminic acid and *N*-glycolylneuraminic acid
	3-O-sulfated heparan sulfate
Cell adhesion and cell–cell contact proteins	α-Dystroglycan
	Coxsackievirus-adenovirus receptor (CAR; Ig superfamily)
	CD4 (Ig superfamily)
	CEACAMs (including Bgp1a, Bgp2, and pregnancy-specific glycoprotein)
	Intercellular adhesion molecule type 1 (ICAM-1; Ig superfamily)
	Integrins
	Junction adhesion molecule (JAM)
	Laminin receptor (high affinity)
	MHC class I and β_2-microglobulin
	Neural cell adhesion molecule (NCAM)
	Signaling lymphocyte activation molecule (SLAM or CDw150)
	Vascular adhesion molecule 1 (VCAM-1)
Chemokine receptors and G-protein-coupled receptors	CC chemokines subfamily: CCR1, CCR2, CCR3, CCR8
	CXC chemokines subfamily: CX3CR1, CXCR4 (Fusin), CXCR6 (BONZO)
	G-protein-coupled receptor: GPR1, GPR15 (BOB)
Complement control protein superfamily	CD21 (CR2)
	CD46
	CD55 (DAF)
	CD59
Growth factor receptors	Epidermal growth factor receptor (EGFR)
	Fibroblast growth factor receptor (FGFR)
	Low-affinity nerve growth factor receptor
Low-density lipoprotein receptor-related proteins	Low-density lipoprotein receptor (LDLR)
	Very low-density lipoprotein receptor (VLDLR)

(*continues*)

TABLE II (*continued*)

Receptor class	Cellular structure
	Tva (receptor for subgroup A avian sarcoma and leukosis virus)
High-density lipoprotein receptor-related proteins	SR-BI (scavenger receptor class B type I)
Poliovirus receptor-related proteins	Nectin-1 α (Prr1α, HveC), β (Prr1β, HIgR), and γ (Ig superfamily)
	Nectin-2 α (Prr2α, HveB) and δ (Prr1δ) (Ig superfamily)
	Poliovirus receptor (PVR or CD155; Ig superfamily)
Transporter proteins	Murine cationic amino acid transporter (MCAT-1)
	Phosphate transporter proteins (Pit1 and Pit2)
	RD114/simian type D retrovirus receptor (RDR)
	Xenotropic and polytropic retrovirus receptor (XPR1)
Tumor necrosis factor receptor-related proteins	HveA (HVEM)
	CAR1 (receptor for subgroups B and D of avian leukosis virus)
	TEF (receptor for subgroup E of avian leukosis virus)
Other proteins	Acetylcholine receptor
	Aminopeptidase N (APN or CD13; metalloproteinase)
	β-Adrenergic receptor
	Carboxypeptidase D
	Members of the tetraspanin family: CD9, CD81
	Folate receptor-α
	Glucose regulated protein 78 (GRP78; member of the heat shock protein 70 family)
	Hyaluronidase-2 (HYAL2; tumor suppressor)
	Mannose receptor
	Transferrin receptor (TfR)
	UDP-galactose transporter

[a] Bgp, biliary glycoprotein; CEACAM, carcinoembryonic antigen-cell adhesion molecule; CR, complement receptor; DAF, decay-accelerating factor; HIgR, herpesvirus immunoglobulin-like receptor; Hve, herpesvirus entry protein; HVEM, herpesvirus entry protein mediator; Ig, immunoglobulin; MHC, major histocompatibility complex; Prr, poliovirus receptor related.

acid, and biliary glycoproteins, which belong to the carcinoembryonic antigen family of the immunoglobulin superfamily. Some of these receptors are shared by viruses associated with different pathologies (Table II).

At least three different receptors have been proposed to be involved in the entry of human hepatitis C virus (HCV) into liver cells: CD81, a member of the tetraspanin superfamily of proteins (Pileri *et al*., 1998),

the low-density lipoprotein receptor (LDLR) (Agnello *et al.*, 1999), and the human scavenger receptor class B type I (hSR-BI) (Scarselli *et al.*, 2002) (Table III). Binding of HCV to CD81 is strain specific, and the binding affinity can be modulated by hypervariable sequences in the envelope protein (Roccasecca *et al.*, 2003). Other hepatitis viruses, despite sharing a specificity for hepatocytes as their main target cells, employ other receptor molecules. The human hepatitis A virus receptor HAVcr-1 is a mucine-like class I integral membrane glycoprotein, and the receptor for duck hepatitis B virus is the C domain of the carboxypeptidase D, gp180 (Urban *et al.*, 2000).

As in the case of viruses with liver tropism, different viruses sharing preferences for other tissues (neural, lymphoid, etc.) do not generally use the same receptor sites. Each class of tissue offers a variety of molecules that can potentially act as virus receptors, and viruses make use of them presumably as the result of ancestral evolutionary processes. As discussed in Section V, analysis of the complete genomic nucleotide sequences of several prokaryotic and eukaryotic organisms, including the first draft of the human genome, has unveiled the presence of several types of mobile genetic elements, suggesting ancestral exchanges of modules among cells, in which viruses likely played an active role. Our present observations on how viruses can penetrate cells and replicate in them are just a snapshot that reflects an instant out of eons of fluid exchanges among cells and autonomous replicons (Bushman, 2002).

The presence on the cell surface of a protein that has been identified as the receptor for a given virus may not be sufficient for a productive viral infection, and there may be multiple mechanisms behind such restrictions: functional domains of the receptor may be blocked in some cellular context, additional proteins (or other cofactors) may be needed, or cells may exhibit impediments for completion of the infection cycle, despite an initial successful interaction with a functional receptor. Mice that are transgenic for the functional form of the poliovirus receptor (PVR or CD155) become susceptible to poliovirus and, upon infection, develop the typical limb paralysis. However, the tissue and organ distribution of the PVR mRNA does not correspond to the sites where virus replicates (Nomoto *et al.*, 1994; Ren and Racaniello, 1992; Ren *et al.*, 1990). Likewise, the *N*-acetylneuraminic acid (sialic acid), which is the receptor for human influenza virus, is common on glycosylated molecules on cell surfaces. As a consequence, influenza viruses can bind to many cell types and yet productive infection occurs generally in the epithelial cells of the respiratory tract when the virus causes disease.

TABLE III

CELL SURFACE MOLECULES PROPOSED TO BE INVOLVED IN VIRUS ENTRY PROCESSES

Virus[a]	Cell surface molecules[b]
Double-stranded DNA viruses	
Adenoviridae	
Human adenovirus	CAR (except subgroup B Ad3 and Ad7; and subgroup D Ad8 and Ad37) (Bergelson *et al*., 1997; Tomko *et al*., 1997; Roelvink *et al*., 1998; 1999; Bewley *et al*., 1999; Freimuth *et al*., 1999); murine CAR (Tomko *et al*., 1997; Bergelson *et al*., 1998); integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ (Wickham *et al*., 1993; Mathias *et al*., 1998; Chiu *et al*., 1999), $\alpha_v\beta_1$ (Li *et al*., 2001); MHC class I (α2 domain), but not allele HLA-A*0201 (Hong *et al*., 1997; Davison *et al*., 1999); HS-GAG (Dechecchi *et al*., 2000, 2001)
Human adenovirus 8, 19a, 37	Sialic acid (α2,3-linked) (Arnberg *et al*., 2000a, 2000b, 2002)
Canine adenovirus 2	CAR (Soudais *et al*., 2000)
Avian adenovirus CELO	CAR (Tan *et al*., 2001)
Herpesviridae, alphaherpesvirinae	
HSV-1/HHV-1 HSV-2/HHV-2	HS-GAG and CS-GAG (WuDunn and Spear, 1989; Shieh *et al*., 1992; Banfield *et al*., 1995); 3-O-sulfated HS (HSV-1 only) (Shukla *et al*., 1999a; Trybala *et al*., 2000); HveA/HVEM (Montgomery *et al*., 1996; Whitbeck *et al*., 1997; Mauri *et al*., 1998); nectin-1 alpha/Prr1α/HveC, beta/Prr1β/HIgR, and gamma (Cocchi *et al*., 1998; Geraghty *et al*., 1998; Lopez *et al*., 2001; Milne *et al*., 2001); nectin-2 alpha/Prr2α/HveB and delta/Prr1δ (HSV-1 mutant Rid and HSV-2) (Warner *et al*., 1998; Lopez *et al*., 2000); murine HveA and HveC (Menotti *et al*., 2000); porcine HveC (Milne *et al*., 2001)
VZV/HHV-3	HS-GAG (Zhu *et al*., 1995); manose-6-phosphate-dependent receptor (Zhu *et al*., 1995)
BHV-1	HS-GAG (Okazaki *et al*., 1991); nectin-1 alpha/Prr1α/HveC and beta/Prr1β/HIgR (Geraghty *et al*., 1998; Spear *et al*., 2000); porcine HveC (Milne *et al*., 2001); murine HveC (Menotti *et al*., 2000); PVR/CD155 (Geraghty *et al*., 1998)

(*continues*)

TABLE III (*continued*)

Virus	Cell surface molecules
PrV	HS-GAG (Mettenleiter *et al.*, 1990); nectin-1 alpha/Prr1α/HveC (Geraghty *et al.*, 1998); nectin-2 alpha/Prr2α/HveB (Warner *et al.*, 1998); murine HveB (Shukla *et al.*, 1999b); porcine HveC (Milne *et al.*, 2001); murine HveC (Menotti *et al.*, 2000); PVR/CD155 (Geraghty *et al.*, 1998)
Herpesviridae, betaherpesvirinae	
HCMV/HHV-5	HS-GAG (Compton *et al.*, 1993); APN/CD13 (Soderberg *et al.*, 1993); MHC class I via β_2-microglobulin (Grundy *et al.*, 1987)
HHV-6	CD46 (Santoro *et al.*, 1999)
HHV-7	HS-GAG (Secchiero *et al.*, 1997; Skrincosky *et al.*, 2000); CD4 (Lusso *et al.*, 1994)
KSHV/HHV-8	HS-GAG (Akula *et al.*, 2001; Birkmann *et al.*, 2001; Wang *et al.*, 2001); integrin $\alpha_3\beta_1$ (Akula *et al.*, 2002)
MCMV	MHC class I (H-2Dd and Kb) (Wykes *et al.*, 1993)
Herpesviridae, gammaherpesvirinae	
EBV/HHV-4	CD21/CR2 (Fingeroth *et al.*, 1984; Frade *et al.*, 1985; Nemerow *et al.*, 1985); MHC class II (HLA-DR, -DP, -DQ) (Reisert *et al.*, 1985; Li *et al.*, 1997; Haan *et al.*, 2000; Haan and Longnecker, 2000)
BHV-4	HS-GAG (Vanderplasschen *et al.*, 1993)
Papovaviridae	
Papillomavirus	α6 integrins (Evander *et al.*, 1997; McMillan *et al.*, 1999); HS-GAG (Giroglou *et al.*, 2001)
Murine polyomavirus	Sialic acid (*N*-acetyl neuraminic acid) (Stehle *et al.*, 1994; Stehle and Harrison, 1996); integrin $\alpha 4\beta 1$ (Caruso *et al.*, 2003)
SV40	MHC class I (Atwood and Norkin, 1989; Breau *et al.*, 1992)
Poxviridae	
Vaccinia virus	HS-GAG and CS-GAG (Chung *et al.*, 1998; Hsiao *et al.*, 1999)
Myxoma virus	CCR1, CCR5, CXCR4 (Lalani *et al.*, 1999)
Single-stranded DNA viruses	
Parvoviridae	
AAV2	HS-GAG (Summerford and Samulski, 1998; Qiu *et al.*, 2000); FGFR 1 (Qing *et al.*, 1999); integrin $\alpha_c\beta_5$ (Summerford *et al.*, 1999)

(*continues*)

TABLE III (*continued*)

Virus	Cell surface molecules
Human virus B19	Erytrocyte P antigen (globoside) (Brown *et al*., 1993; Chipman *et al*., 1996)
Bovine parvovirus	Sialic acid, glycophorin A (Thacker and Johnson, 1998)
Canine parvovirus	Sialic acid (Barbis *et al*., 1992); human, feline and canine TfR (Parker *et al*., 2001; Hueffer *et al*., 2003)
Feline parvovirus	Sialic acid (Barbis *et al*., 1992); human and feline TfR (Parker *et al*., 2001)
DNA and RNA reverse transcribing viruses	
Hepadnaviridae	
HBV	Sialic acid (Komai *et al*., 1988); asialoglycoprotein receptor (Treichel *et al*., 1994); endonexin II (Hertogs *et al*., 1993)
Duck hepatitis B virus	Carboxypeptidase D (Breiner *et al*., 1998; Urban *et al*., 1998; Tong *et al*., 1999; Urban *et al*., 2000)
Retroviridae (alpharetrovirus)	
ALV-A	Tva (Bates *et al*., 1993; Connolly *et al*., 1994)
ALV-B, -D	CAR1 (Brojatsch *et al*., 1996; Adkins *et al*., 2001)
ALV-E	TEF (Adkins *et al*., 1997)
Retroviridae (betaretrovirus)	
MMTV	MTVR/mouse TfR-1 (Ross *et al*., 2002)
JSRV, ONAV	HYAL2 (Rai *et al*., 2001; Miller 2003)
SRV (receptor group 1)	RDR/R-receptor/ATB^0/ASCT2/Na(+)-dependent neutral amino acid transporter type 2 (Kewalramani *et al*., 1992; Koo *et al*., 1994; Rasko *et al*., 1999; Tailor *et al*., 1999b)
Retroviridae (gammaretrovirus)	
BaEV (receptor group 1)	RDR/R-Receptor/ATB^0/ASCT2 /Na(+)-dependent neutral amino acid transporter type 2 (Kewalramani *et al*., 1992; Koo *et al*., 1994; Rasko *et al*., 1999; Tailor *et al*., 1999b)
FeLV-B (receptor group 5)	Pit1/Glvr (Takeuchi *et al*., 1992; Miller, 1996); Pit2 /Ram-1/Glvr-2 (mutants of FeLV-B only) (Sugai *et al*., 2001)
FeLV-C (receptor group 4)	FLVCR (Tailor *et al*., 1999c)
FeLV-T	Pit1/Glvr; FeLIX (Anderson *et al*., 2000; Lauring *et al*., 2001)
GALV (receptor group 5)	Pit1/Glvr (O'Hara *et al*., 1990; Miller and Miller, 1994; Miller, 1996)

(*continues*)

TABLE III (*continued*)

Virus	Cell surface molecules
MLV-A (receptor group 2)	Pit2 /Ram-1/Glvr-2 (Miller *et al*., 1994; Miller and Miller, 1994; van Zeijl *et al*., 1994)
MLV-10A1	Pit1/Glvr; Pit2 /Ram-1/Glvr-2 (Miller and Miller, 1994; Miller, 1996)
MLV-E	MCAT-1/REC-1 /ecoR/ATRC-1 (Albritton *et al*., 1989; Wang *et al*., 1991a)
MLV-X (receptor group 3)	XPR1 /X-receptor/Rmc-1/sxv (Battini *et al*., 1999; Tailor *et al*., 1999a; Yang *et al*., 1999)
P-MLV (receptor group 3)	XPR1 /X-receptor/Rmc-1/sxv (Battini *et al*., 1999; Tailor *et al*., 1999a; Yang *et al*., 1999)
RD114 (receptor group 1)	RDR/R-receptor/ATB^0/ASCT2 /Na(+)-dependent neutral amino acid transporter type 2 (Kewalramani *et al*., 1992; Koo *et al*., 1994; Rasko *et al*., 1999; Tailor *et al*., 1999b)
REV (receptor group 1)	RDR/R-receptor/ATB^0/ASCT2 /Na(+)-dependent neutral amino acid transporter type 2 (Kewalramani *et al*., 1992; Koo *et al*., 1994; Rasko *et al*., 1999; Tailor *et al*., 1999b)
Retroviridae (deltaretrovirus)	
BLV (receptor group 6)	BLVRcp1/Blvr (Ban *et al*., 1993)
HTLV-I (receptor group 7)	MHC class I; interleukin 2 receptor (Clarke *et al*., 1983; Lando *et al*., 1983; Kohtz *et al*., 1988)
Retroviridae (lentiviruses)	
FIV-A, -B	CXCR4/fusin (Willett *et al*., 1997; Poeschla *et al*., 1998; Richardson *et al*., 1999; Frey *et al*., 2001); feline homologue of CD9 (Willett *et al*., 1994)
HIV-1 (receptor group 8)	HS-GAG (Patel *et al*., 1993; Roderiquez *et al*., 1995; Saphire *et al*., 2001); CD4 (Dalgleish *et al*., 1984; Klatzmann *et al*., 1984; Maddon *et al*., 1986; McDougal *et al*., 1986); CXCR4/Fusin; CCR5/CC-CKR5; CCR2b/CC-CKR2b; CCR3/CC-CKR3; CCR8/Chem-1; BOB/GPR15; Bonzo/CXCR6/STRL33/TYMSRT; GPR1; APJ (Alkhatib *et al*., 1996; Choe *et al*., 1996; Deng *et al*., 1996, 1997; Doranz *et al*., 1996; Dragic *et al*., 1996; Feng *et al*., 1996; Lapham *et al*., 1996; Liao *et al*., 1997; Loetscher *et al*., 1997; Rucker *et al*., 1997; Choe *et al*., 1998; Edinger *et al*., 1998a,b; Shimizu *et al*., 1999); US28 (Pleskoff *et al*., 1997; Rucker *et al*., 1997); BLTR (Owman *et al*., 1998); CD8 (Saha *et al*., 2001); GalCer (Bhat *et al*., 1991; Fantini *et al*., 1993); integrin $\alpha_v\beta_3$ (Lafrenie *et al*., 2002)

(*continues*)

TABLE III (*continued*)

Virus	Cell surface molecules
HIV-2 (receptor group 8)	CD4; CXCR4/fusin; CCR5/CC-CKR5; CCR8/Chem-1; GPR1 (Owen *et al*., 1998; Reeves *et al*., 1999; Shimizu *et al*., 1999; Liu *et al*., 2000); GalCer (Hammache *et al*., 1998); US28 (Pleskoff *et al*., 1997)
SIV (receptor group 8)	CD4; CCR3/CC-CKR3; CCR5/CC-CKR5; CCR8/Chem-1; BOB/GPR15; Bonzo/CXCR6/STRL33/TYMSRT; GPR1; APJ (Alkhatib *et al*., 1997; Deng *et al*., 1997; Farzan *et al*., 1997; Rucker *et al*., 1997; Choe *et al*., 1998; Edinger *et al*., 1998b; Liu *et al*., 2000)
Visna virus	Ovine MHC class II (Dalziel *et al*., 1991)
Double-stranded RNA Viruses	
Reoviridae	
Reovirus type 1	JAM (Barton *et al*., 2001a, 2001b); carbohydrate (unknown nature) (Lerner *et al*., 1963; Chappell *et al*., 2000); EGFR (Strong *et al*., 1993; Tang *et al*., 1993)
Reovirus type 3	JAM (Barton *et al*., 2001a, 2001b); sialic acid (Gentsch and Pacitti, 1987; Paul *et al*., 1989; Chappell *et al*., 2000); type A glycophorin (Paul and Lee, 1987); β-adrenergic receptor (Co *et al*., 1985b; Choi and Lee, 1988; Donta and Shanley, 1990)
Group A human rotavirus (strain Wa)	Integrins $\alpha_v\beta_3$ (Guerrero *et al*., 2000); $\alpha_2\beta_1$ (VLA-2) (Ciarlet *et al*., 2002)
Group A simian rotavirus (strains SA-11 and RRV)	Sialic acid (not RRV variant nar 3) (Yolken *et al*., 1987; Willoughby and Yolken, 1990; Mendez *et al*., 1993, 1999; Ciarlet and Estes, 1999), gangliosides (Superti and Donelli, 1991; Srnka *et al*., 1992; Delorme *et al*., 2001); integrins $\alpha_2\beta_1$ (VLA-2), $\alpha_4\beta_1$ (VLA-4) (Hewish *et al*., 2000; Zarate *et al*., 2000; Ciarlet *et al*., 2002), $\alpha_v\beta_3$ (Guerrero *et al*., 2000)
Group A porcine rotavirus (strain OSU)	Gangliosides (Rolsma *et al*., 1994, 1998); integrins $\alpha_2\beta_1$ (VLA-2) (Coulson *et al*., 1997; Ciarlet *et al*., 2002)
Negative-sense, single-stranded RNA viruses	
Arenaviridae	
LCMV	α-Dystroglycan (Cao *et al*., 1998; Kunz *et al*., 2001)
Lassa fever virus	α-Dystroglycan (Cao *et al*., 1998)
New World arenavirus (clade C, but not clade A and B viruses)	α-Dystroglycan (Spiropoulou *et al*., 2002)

(*continues*)

TABLE III (*continued*)

Virus	Cell surface molecules
Bunyaviridae	
Hantaviruses	β3 integrins (Gavrilovskaya *et al.*, 1998, 1999)
Filoviridae	
Ebola virus	FR-α (Chan *et al.*, 2001)
Marburg virus	FR-α (Chan *et al.*, 2001); asialoglycoprotein receptor (Becker *et al.*, 1995)
Orthomyxoviridae	
Influenza A, B	Sialic acid (*N*-acetylneuraminic acid, α2,3 and α2,6-linked) (Paulson *et al.*, 1979; Weis *et al.*, 1988; Eisen *et al.*, 1997); mannose receptor (Reading *et al.*, 2000)
Influenza C	Sialic acid (*N*-acetyl-9-*O*-acetylneuraminic acid) (Herrler *et al.*, 1985; Rosenthal *et al.*, 1998)
Paramyxoviridae, Paramyxovirinae	
Human parainfluenza virus 1, 3	Sialic acid residues on gangliosides glycolipids (Suzuki *et al.*, 2001)
Measles virus	CD46 (Dorig *et al.*, 1993; Naniche *et al.*, 1993; Schneider-Schaulies *et al.*, 1995; Horvat *et al.*, 1996; Buckland and Wild, 1997; Buchholz *et al.*, 1997; Niewiesk *et al.*, 1997); SLAM (Tatsuo *et al.*, 2000b)
Sendai virus	Sialic acid residues on glycophorin and gangliosides GD1a, GT1b, GQ1b (glycolipids) (Markwell and Paulson, 1980; Wu *et al.*, 1980; Markwell *et al.*, 1981, 1986; Wybenga *et al.*, 1996); asialoglycoprotein receptor (Markwell *et al.*, 1985; Bitzer *et al.*, 1997)
NDV	Sialic acid (Crennell *et al.*, 2000)
Rinderpest virus	SLAM (Tatsuo *et al.*, 2001)
Canine distemper virus	CD9 (Loffler *et al.*, 1997); SLAM (Tatsuo *et al.*, 2001)
Paramyxoviridae, pneumovirinae	
HRSV	Iduronic acid-containing glycosaminoglycans (heparan sulfate and chondroitin sulfate B) (Feldman *et al.*, 1999; Hallak *et al.*, 2000; Martinez and Melero, 2000); CX3CR1 (Tripp *et al.*, 2001)
Rhabdoviridae	
Rabies virus	Acetylcholine receptor (Lentz *et al.*, 1982, 1984; Hanham *et al.*, 1993; Gastka *et al.*, 1996); NCAM (Thoulouze *et al.*, 1998); low-affinity nerve growth factor receptor p75NTR (Tuffereau *et al.*, 1998, 2001); sialylated gangliosides

(*continues*)

TABLE III (*continued*)

Virus	Cell surface molecules
	(Superti *et al*., 1986); phospholipids (Superti *et al*., 1984)
VSV	Phosphatidylserine (Schlegel *et al*., 1983); phosphatidylinositol (Mastromarino *et al*., 1987)
Positive-sense, single-stranded RNA viruses	
Arteriviridae	
Lactate dehydrogenase virus	Mouse Ia antigens (Inada and Mims, 1984)
Coronaviridae	
HCoV-229E	Human APN (CD13) (Yeager *et al*., 1992; Lachance *et al*., 1998); feline APN (Tresnan *et al*., 1996)
HCoV-OC43	Sialic acid (*N*-acetyl-9-*O*-acetylneuraminic acid) (Vlasak *et al*., 1988)
TGEV	Porcine APN (Delmas *et al*., 1992); feline APN (Tresnan *et al*., 1996); canine APN (Benbacer *et al*., 1997); bovine APN (Benbacer *et al*., 1997); sialic acid (*N*-glycolylneuraminic acid) (Schultze *et al*., 1995, 1996)
BCV	Sialic acid (*N*-acetyl-9-*O*-acetylneuraminic acid) (Vlasak *et al*., 1988; Schultze *et al*., 1991; Schultze and Herrler, 1992)
FIPV	Feline APN (Tresnan *et al*., 1996); canine APN (Benbacer *et al*., 1997)
CCV	Canine APN (Benbacer *et al*., 1997); feline APN (Tresnan *et al*., 1996)
MHV	CEACAMs (Dveksler *et al*., 1991; Williams *et al*., 1991a; Compton *et al*., 1992; Yokomori and Lai, 1992; Dveksler *et al*., 1993; Chen *et al*., 1997a), including biliary glycoproteins (Bgp1a, Bgp2) (Nedellec *et al*., 1994) and pregnancy-specific glycoprotein (Chen *et al*., 1995)
Flaviviridae	
Dengue virus	HS-GAG (Chen *et al*., 1997b; Germi *et al*., 2002)
Yellow fever virus	HS-GAG (Germi *et al*., 2002)
HCV	LDLR protein family (Agnello *et al*., 1999; Monazahian *et al*., 1999); CD81 (Pileri *et al*., 1998; Flint *et al*., 1999); tamarin CD81 (Allander *et al*., 2000); human SR-BI (Scarselli *et al*., 2002)
BVDV	LDLR protein family (Agnello *et al*., 1999)
GB virus C/hepatitis G virus	LDLR protein family (Agnello *et al*., 1999)
CSFV	HS-GAG (Hulst *et al*., 2000, 2001)

(*continues*)

TABLE III (*continued*)

Virus	Cell surface molecules
Picornaviridae (aphthovirus)	
FMDV	HS-GAG (Jackson *et al.*, 1996; Sa-Carvalho *et al.*, 1997; Fry *et al.*, 1999; Baranowski *et al.*, 2000); integrins $\alpha_v\beta_3$ (Fox *et al.*, 1989; Berinstein *et al.*, 1995; Jackson *et al.*, 1997; Neff *et al.*, 1998), $\alpha_v\beta_6$ (Jackson *et al.*, 2000b), $\alpha_v\beta_1$ (Jackson *et al.*, 2002), $\alpha_5\beta_1$ (Jackson *et al.*, 2000a)
Picornaviridae (cardiovirus)	
EMCV	VCAM-1 (Huber, 1994); sialylated glycophorin A (Allaway and Burness, 1986)
Picornaviridae (enterovirus)	
Coxsackievirus A9	Integrin $\alpha_v\beta_3$ (Roivainen *et al.*, 1991; Roivainen *et al.*, 1994; Berinstein *et al.*, 1995; Triantafilou *et al.*, 1999); GRP78 (Triantafilou *et al.*, 2002); MHC class I via β_2-microglobulin (Triantafilou *et al.*, 1999)
Coxsackievirus B1-6	CAR (Bergelson *et al.*, 1997; Shafren *et al.*, 1997c; Tomko *et al.*, 1997; Martino *et al.*, 2000; He *et al.*, 2001); murine CAR (Tomko *et al.*, 1997; Bergelson *et al.*, 1998)
Coxsackievirus B1, B3, B5	DAF (Bergelson *et al.*, 1995; Shafren *et al.*, 1995; Martino *et al.*, 1998); integrin $\alpha_v\beta_6$ (Agrez *et al.*, 1997)
Coxsackievirus A21	DAF (Shafren *et al.*, 1997b); ICAM-1 (Shafren *et al.*, 1997a,b; 1997b; Xiao *et al.*, 2001)
Echovirus 1, 8	Integrin $\alpha_2\beta_1$ (VLA-2) (Bergelson *et al.*, 1992, 1993; King *et al.*, 1995)
Echovirus 9Barty	Integrin $\alpha_v\beta_3$ (Nelsen-Salz *et al.*, 1999)
Echovirus 3, 6, 7, 11–13, 20, 21, 24, 29, 33	DAF (Bergelson *et al.*, 1994; Ward *et al.*, 1994; Powell *et al.*, 1997); MHC class I via β_2-microglobulin (Ward *et al.*, 1998); HS-GAG (Goodfellow *et al.*, 2001); CD59 (Goodfellow *et al.*, 2000)
Enterovirus 70	DAF (Karnauchow *et al.*, 1996)
TMEV strains BeAn, DA	Sialic acid (Zhou *et al.*, 1997, 2000)
TMEV strain GDVII	UDP-galactose transporter (or galactose containing glycoprotein) (Hertzler *et al.*, 2001); HS-GAG (Reddi and Lipton, 2002)
Poliovirus 1–3	PVR (Mendelsohn *et al.*, 1989; Racaniello, 1996; Belnap *et al.*, 2000; He *et al.*, 2000)
Swine vesicular disease virus	CAR (Martino *et al.*, 2000)

(*continues*)

TABLE III (*continued*)

Virus	Cell Surface Molecules
Picornaviridae (Hepatovirus)	
Hepatitis A virus	HAVcr-1 (Kaplan *et al*., 1996; Feigelstock *et al*., 1998)
Picornaviridae (Rhinovirus)	
Major group HRV	ICAM-1 (Greve *et al*., 1989; Staunton *et al*., 1989; Tomassini *et al*., 1989; Olson *et al*., 1993; Kolatkar *et al*., 1999)
Minor group HRV	VLDLR (Hofer *et al*., 1994; Marlovits *et al*., 1998; Hewat *et al*., 2000); avian homologues of the mammalian LDLR (Gruenberger *et al*., 1995)
HRV 87	Sialic acid (Uncapher *et al*., 1991)
Picornaviridae (Parechovirus)	
Human parechovirus 1/echovirus 22	Integrins $\alpha_v\beta_1$ and $\alpha_v\beta_3$ (Triantafilou *et al*., 2000)
Togaviridae	
Sindbis virus	High-affinity laminin receptor (Wang *et al*., 1992); HS-GAG (Byrnes and Griffin, 1998; Klimstra *et al*., 1998)

[a] Abbreviations of virus names: AAV2, adeno-associated virus type 2; Ad, adenovirus; ALV, avian leukosis virus; BaEV, baboon endogenous retrovirus; BCV, bovine coronavirus; BHV, bovine herpesvirus; BLV, bovine leukemia virus; BVDV, bovine viral diarrhea virus; CCV canine coronavirus; CSFV classical swine fever virus; EBV, Epstein–Barr virus; EMCV, encephalomyocarditis virus; FeLV, feline leukemia virus; FIPV, feline infectious peritonitis virus; FIV, feline immunodeficiency virus; FMDV, foot-and-mouth disease virus; GALV, gibbon ape leukemia virus; HBV, hepatitis B virus; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HCoV, human coronavirus; HHV, human herpes virus; HIV, human immunodeficiency virus; HRSV, human respiratory syncytial virus; HRV, human rhinovirus; HSV, herpes simplex virus; HTLV, human T-cell leukemia virus; JSRV, Jaagsiekte sheep retrovirus; KSHV, Kaposi's sarcoma-associated herpesvirus; LCMV, lymphocytic choriomeningitis virus; MCMV, murine cytomegalovirus; MHV, mouse hepatitis virus; MLV-A, amphotropic murine leukemia virus; MLV-E, ecotropic murine leukemia virus; MLV-X, xenotropic murine leukemia virus; MMTV, mouse mammary tumor virus; NDV, Newcastle disease virus; ONAV, ovine nasal adenocarcinoma virus; P-MLV, polytropic murine leukemia virus; PrV, pseudorabies virus; RD114, cat endogenous retrovirus; REV, reticuloendotheliosis virus; SIV, simian immunodeficiency virus; SRV, simian retroviruse; SV40, simian virus 40; TEF, name given to the receptor for subgroup E of avian leukosis virus; TGEV, transmissible gastroenteritis virus; TMEV, Theiler's encephalomyelitis virus; VSV, vesicular stomatitis virus; VZV, varicella zoster virus.

[b] Abbreviations of cellular structures: APJ, name given to a ligand-unknown "orphan" seven transmembrane domain receptor of the central nervous system; APN, aminopeptidase N; BLTR, leukotriene B4 receptor; BLVR, bovine leukemia virus receptor; Bgp, biliary glycoprotein; CAR, coxsackievirus-adenovirus receptor; CAR1, name given to the receptor for subgroups B and D avian leukosis virus; CCR, CC-chemokine receptor; CEACAM, carcinoembryonic antigen-cell adhesion molecule; CR, complement receptor; CS-GAG, chondroitin sulfate glycosaminoglycan; CXCR, CXC-chemokine receptor; DAF,

A. A Virus May Use Different Receptor Types

Some viruses apparently use only a single receptor to infect their target cells, whereas others are able to exploit several alternative receptors to initiate their replication in different cell lines or even to enter the same cell type (Table III). Evidence shows that foot-and-mouth disease virus (FMDV) may employ integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_6$ as receptors. Recognition of these integrins is dependent on an Arg-Gly-Asp (RGD) triplet found in the surface G-H loop of capsid protein VP1, and this triplet is very conserved among natural isolates of the seven FMDV serotypes (Domingo *et al*., 1990, 1992; Mateu, 1995). However, several studies suggest the use of alternative receptors by FMDV, both in cell culture and *in vivo*. Upon adaptation of FMDV to cell culture, mutations may occur that result in the acquisition of positively charged amino acids at key residues of the capsid surface that allow FMDV to enter cells via heparan sulfate (HS) and possibly other receptors (Baranowski *et al*., 1998, 2000; Escarmis *et al*., 1998; Fry *et al*., 1999; Jackson *et al*., 1996; Sa-Carvalho *et al*., 1997; Sevilla *et al*., 1998; Zhao *et al*., 2003). Following this seminal observation with FMDV, studies with other viruses have demonstrated that passage in cell culture results in evolutionary changes that allow the viruses to use HS for cell entry. Examples include Sindbis virus adapted to BHK cells (Klimstra *et al*., 1998), classical swine fever virus propagated in swine kidney cells (Hulst *et al*., 2000), and variants of human rhinovirus 89 adapted to HEp2 cells (Reischl *et al*., 2001; Vlasak *et al*., 2002) (Section II,B).

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decay-accelerating factor; EGFR, epidermal growth factor receptor; FeLIX, FeLV infectivity X-essory protein; FLVCR, feline leukemia virus subgroup C receptor; FGFR, fibroblast growth factor receptor; FR-α, folate receptor-α; GalCer, galactosylceramide; Glvr, gibbon ape leukemia virus receptor; GPR, G protein-coupled receptor; GRP, glucose-regulated protein; HAVcr, hepatitis A virus cellular receptor 1; HIgR, herpesvirus immunoglobulin-like receptor; HS-GAG, heparan sulfate glycosaminoglycan; HLA, human leukocyte antigen; Hve, herpesvirus entry protein; HVEM, herpesvirus entry protein mediator; HYAL, hyaluronidase; ICAM-1, intercellular adhesion molecule type 1; JAM, junction adhesion molecule; LDLR, low-density lipoprotein receptor; MCAT, murine cationic amino acid transporter; MHC, major histocompatibility complex; MTVR, mouse mammary tumor virus receptor; NCAM, neural cell adhesion molecule; Prr, poliovirus receptor related; PVR, poliovirus receptor; SLAM, signaling lymphocyte activation molecule; SR-BI, scavenger receptor class B type I; RDR, name given to the RD114/simian type D retrovirus receptor; TfR, transferrin receptor; Tva, name given to the receptor for subgroup A avian sarcoma and leukosis virus; Pit, inorganic phosphate transporter; VCAM, vascular adhesion molecule; VLA, very late antigen; VLDLR, very low-density lipoprotein receptor; XPR1, xenotropic and polytropic retrovirus receptor.

A well-documented case of use of multiple receptors is that of lentiviruses HIV-1, HIV-2, and simian immunodeficiency virus (SIV). Almost all strains described to date require interactions with two molecules on the cell surface. One of them acts as an attachment receptor, with which viral glycoprotein gp120 interacts. This interaction promotes conformational changes that expose the fusogenic peptide of gp41, which, in turn interacts with the fusion receptor. This is essential for the fusion of viral and cellular membranes and therefore allows the viral capsid to enter the host cell. The group of cellular molecules involved in lentivirus fusion are referred to as coreceptors. It has been suggested that those strains shown to use only the fusion receptor have already undergone, at least partially, the conformational changes normally induced by the attachment receptor (Berger *et al*., 1999).

The helper T-cell differentiation antigen CD4 is an important attachment receptor for HIV-1, HIV-2, and SIV, but other cell surface molecules, including galactosyl ceramide (GalCer), syndecans, and other glycosaminoglycans, have been found to play a role in the attachment of these viruses (Baba *et al*., 1988; Ito *et al*., 1987; Saphire *et al*., 2001; Zhang *et al*., 2002). The preferential use of some attachment receptors over others is thought to be influenced by their relative abundance on the cell surface. Thus, on macrophages, CD4 is scarce and HIV attachment takes place mainly through HS proteoglycans, especially syndecans (Saphire *et al*., 2001). It has been reported that integrin $\alpha_v\beta_3$ is involved in the infection of macrophages that have differentiated *in vitro* (Lafrenie *et al*., 2002); certainly, the significance of this interaction *in vivo* deserves further study. HIV infection of colon epithelia *in vivo* is CD4–independent and is believed to take place through GalCer (Bhat *et al*., 1993; Fantini *et al*., 1993). Neuronal cells and intestinal epithelia are rarely infected *in vivo*, but binding of HIV envelope glycoproteins to GalCer contributes to the selection of CCR5-binding variants following vertical transmission (Meng *et al*., 2002). This GalCer-mediated selection may also contribute to pathogenesis, e.g., in the gastrointestinal disorders associated with HIV infection and in neuronal dysfunction in AIDS dementia. HIV populations that acquired the capacity to bind CD8 and replicate in $CD8^+$ T cells have been recovered from a patient at a late stage of the infection (Saha *et al*., 2001). A wide variety of molecules from different structural and functional families can act as fusion receptors (or coreceptors). The most important is the chemokine receptor superfamily, for many of its members can bind HIV and/or SIV. A related protein is a β-chemokine receptor US28, encoded by human cytomegalovirus (Pleskoff *et al*., 1997). There are also other coreceptors, such as the

leukotriene B_4 receptor (BLTR) (which has structural homology with chemokine receptors) (Owman *et al.*, 1998) and an orphan receptor of the central nervous system (APJ). Usually, most HIV strains evolve *in vivo* from using mainly CCR5 (R5 viruses) to using mainly CXCR4 (X4 viruses), but many other coreceptors can be used simultaneously.

An example in which the functional receptor for a virus is a complex of several cell molecules that the virus uses in a multistep process is found among *Reoviridae* (Mendez *et al.*, 1999). Rotaviruses are important viral agents of acute gastroenteritis in young children and in many animal species. Rotavirus strains differ in their requirement of sialic acid for initial binding to the cell surface: a minority of animal rotaviruses require the presence of sialic acid residues on the cell surface for efficient binding and infectivity (Arias *et al.*, 2001; Zarate *et al.*, 2000), but most animal and human rotaviruses do not. However, binding to sialic acid is not an essential step for rotavirus infection, as confirmed by the isolation of sialic-independent variants from a sialic-dependent strain (Mendez *et al.*, 1993). The existence of a postattachment cell receptor common to most, if not all, rotavirus strains has been proposed. Integrin $\alpha_v\beta_3$ blocks the infectivity, although not the binding, of the sialic acid-dependent rhesus rotavirus (RRV), its sialic acid-independent mutant nar3, and the sialic acid-independent human rotavirus strain WA, suggesting that $\alpha_v\beta_3$ interacts with both neuraminidase-sensitive and neuraminidase-resistant rotaviruses at a postbinding, probably penetration, step (Guerrero *et al.*, 2000). Other integrins, such as $\alpha_2\beta_1$, $\alpha_4\beta_1$, and $\alpha_x\beta_2$, have also been reported to play a role in rotavirus entry (Ciarlet *et al.*, 2002; Coulson *et al.*, 1997; Hewish *et al.*, 2000; Londrigan *et al.*, 2000).

Cell recognition by herpesviruses offers a classical example of complexity in virus–receptor interactions. The initial contact of herpes simplex virus 1 (HSV-1), the prototype of the *Alphaherpesvirinae* subfamily, with cells is usually the binding of virus to HS proteoglycans (Shieh *et al.*, 1992; Shukla and Spear, 2001; WuDunn and Spear, 1989), mediated by glycoprotein gC and, to a lesser extent, by gB. Then, glycoprotein gD can interact with at least three different classes of molecules that can act as entry mediators for HSV-1. These molecules include HveA (which belongs to the family of tumor necrosis receptor proteins), nectin-1 (HveC) and nectin-2 (HveB) (two members of the immunoglobulin superfamily), and 3-O-sulfated HS (reviewed in Spear *et al.*, 2000; Shukla and Spear, 2001). The binding of gD to one of these different receptors initiates fusion between the viral envelope and the cell membrane. The related Epstein–Barr virus (EBV), a transforming virus of the *gammaherpesvirinae* subfamily,

displays a marked B-cell lymphotropism associated with the expression of receptor CD21 (or CR2). EBV has been associated with a number of human proliferative diseases, including B-cells lymphomas, a clinical condition encountered in immunocompromised hosts (Krance *et al*., 1999). However, EBV can replicate in differentiated epithelial cells that do not express CD21, suggesting that other receptors may be involved in EBV infections (reviewed in Schneider-Schaulies, 2000).

Studies with vaccinia virus revealed that two different forms of the same virus can bind to different cellular receptors. Intracellular mature virus and extracellular enveloped virus are two antigenically and structurally distinct infectious virions that bind to unidentified and possibly different cellular receptors (Vanderplasschen and Smith, 1997). Other examples of the use of several receptor types by a virus include adenoviruses, coronaviruses, hepatitis C virus, influenza virus, measles virus, and rabies virus (references and additional examples in Table III and Section IV).

B. A Receptor Type Can Be Used by Several Viruses and Other Microbial Pathogens

Early evidence that viruses can share receptors was obtained in studies on interference in cell binding between different viruses (Lonberg-Holm *et al*., 1976). Many receptor types are used by viruses of different families, and several examples can be identified by examining Table III (Baranowski *et al*., 2001). A remarkable example is the shared use of coxsackievirus adenovirus receptor (CAR) by some human and animal adenoviruses and by some picornaviruses of different genera (Bergelson *et al*., 1997). These viruses belong to immeasurably distant families and are associated with unrelated diseases. Adenoviruses 2 and 5 are agents of respiratory disease in children, whereas coxsackieviruses B1 to B6 may be associated with febrile illness, meningitis, and some types of cardiopathies.

Other examples of shared receptors by viruses include cell surface components belonging to a wide range of receptor classes such as cell adhesion and cell–cell contact proteins (CD4, ICAM-1, integrins, MHC I), chemokine receptors (CXCR4), members of the complement control protein superfamily (CD46, DAF), low-density lipoprotein receptor and poliovirus receptor-related proteins, transporter proteins, and aminopeptidase N, as well as extracellular matrix components and sugar derivatives (references and additional examples in Table III and Section IV) (Baranowski *et al*., 2001).

Ubiquitous extracellular matrix components such as HS glycosaminoglycans are the preferential class of receptors employed by many viruses (Table III). The abundance of such molecules at the cell surface may facilitate initial contact with the virus, but subsequent interactions with more specific receptors may be needed. For many viral systems, attachment to HS is not an absolute requirement for infection, and cells devoid of HS can be infected, although with a reduced efficiency. Binding to heparin is a phenotypic trait displayed by several viruses propagated in cell culture, presumably because the interaction with HS residues may provide a selective advantage for viruses evolving in cultured cells (Section IV,B). Microbial pathogens that bind to cell surface HS proteoglycans include numerous intracellular (*Borrelia burgdorferia*, *Chlamydia trachomatis*, *Listeria monocytogenes*, *Mycobacterium* spp., *Neisseria gonorrhoeae*) and extracellular (*Bordetella pertussis*, *Haemophilus influenzae*, *Helicobacter pylori*, *Staphylococcus aureus*, *Streptococcus* spp.) bacteria, fungi, and other cellular parasites (*Leishmania* spp., *Plasmodium* spp., *Trypanosoma cruzi*) (reviewed in Bernfield *et al*., 1999).

Integrins have several features that make them attractive portals of entry for pathogens (reviewed in Krukonis and Isberg, 1997; Mims *et al*., 2001; Parkes and Hart, 2000). Interaction of adenovirus with α_v integrins has been reviewed (Nemerow and Stewart, 1999; Nemerow, 2000) (Table II). A variety of bacterial pathogens also use integrin receptors to either adhere to or enter into host cells. *Yersinia pseudotuberculosis* is a gram-negative enteropathogen, which infects cells of the gut wall via binding to $\beta 1$ integrins (Isberg and Leong, 1990), and *Yersinia enterocolitica* uses integrins to bind to cells of the intestinal ephitelium. The interaction of *Yersinia* with host surface integrins induces activation of the cytoskeleton and the rearrangement of actin into pseudopods that engulf the bacteria (Krukonis and Isberg, 1997). A number of microbial pathogens, including *B. pertussis*, *Coxiella burnetti*, *E. coli*, *Histoplasma capsulatum*, *Legionella pneumophila*, *Leishmania* spp., and *Rhodococcus equi*, make use of integrins $\alpha_M\beta_2$ and $\alpha_x\beta_2$, also known as complement receptors (CR3 and CR4), to enter the macrophage and avoid the host microbicidal oxidative burst (Aderem and Underhill, 1999; Capo *et al*., 1999; Krukonis and Isberg, 1997; see *review* of cell entry mechanisms by diverse pathogens in Mims *et al*., 2001).

Another recently described example of a receptor type used by several microbial pathogens is CD81, a putative receptor for HCV (Pileri *et al*., 1998), which is required on hepatocytes for *Plasmodium falciparum* and *yoelii* sporozoite infectivity (Silvie *et al*., 2003).

C. Virus–Receptor Interactions Revealed by Structural Studies

The specific interactions between a viral protein or glycoprotein and receptor molecules are amenable to structural studies, and such studies are providing essential new information for the understanding of virus–receptor interactions. Enveloped viruses, such as HIV, attach to host cells by means of spike-like membrane glycoproteins, whereas most nonenveloped viruses, such as picornaviruses, attach by means of specialized domains integral to their capsids. Some viruses cannot be assigned to one of the two binding modes. For example, adenoviruses are nonenveloped particles, but have trimeric fibers projecting from the vertices of their icosahedral capsid, which terminate in a globular knob domain responsible for the specific interactions with the cellular receptors (Nemerow, 2000). The interaction of the surface hemagglutinin of human influenza virus with *N*-acetylneuraminic acid and the conformational alterations associated with the pH-dependent membrane fusion stand as one of the best characterized virus entry processes at both functional and structural levels. The entry of human influenza virus into human cells has been reviewed (Skehel and Wiley, 2000) and is not treated here except as an illustration of some concepts in following sections. Viral receptor-binding sites typically comprise highly conserved amino acid residues (Rossmann *et al*., 1985), a requirement that appears to guarantee survival of the virus. Early crystallographic studies of picornaviruses indicated that these and other viruses may shield their receptor-binding sites in cavities or surface depressions ("canyons") that are inaccessible to antibody molecules, and this inaccessibility would confer viruses a selective advantage (Rossmann *et al*., 1985). Subsequent studies with other viruses have shown that receptor-binding sites may also occur in highly exposed regions of the viral surface (He *et al*., 2002; Hewat *et al*., 2000; Verdaguer *et al*., 1995). It may be predicted that when receptor-binding sites and neutralizing antibody-binding sites overlap at the virus surface, amino acid replacements needed for antibody escape may be deleterious as they affect receptor recognition. However, in line with the dynamics of RNA virus populations (Section II,B), this conflict is just one of the several instances in which negative selection may act to maintain variants at low levels and to rescue fit viruses through compensatory mutations. There is no strict requirement that surface residues involved in receptor recognition be entirely shielded from immune attack. Another proposed mechanism to compensate for possible adverse effects of amino acid replacements regarding cell

recognition is provided by the water buffer hypothesis, discussed at the end of this section.

Cryoelectron microscopy (cryo-EM) and image reconstruction methods allow direct visualization of virus–receptor complexes, which are usually too large and unstable to be amenable to analysis by high-resolution X-ray crystallography. In addition, when high-resolution structures of the virus and receptor domains are known, a pseudo-atomic model of the virus–receptor complex can be reproduced by docking the atomic structures together using the cryo-EM density map as a guide. Cryo-EM analyses of the major group rhinoviruses 16 and 14 (HRV16, HRV14) in complex with intracellular adhesion molecule-1 (ICAM-1 or CD54) (Kolatkar *et al*., 1999; Olson *et al*., 1993) and of the enteroviruses poliovirus type 1 Mahoney (PV1) in complex with poliovirus receptor (PVR or CD155) (Belnap *et al*., 2000; He *et al*., 2000; Xing *et al*., 2000), coxsackievirus A21 (CAV21) in complex with ICAM-1 (Xiao *et al*., 2001) and coxsackievirus B3 (CBV3) in complex with the coxsackie-adenovirus receptor (CAR) (He *et al*., 2001) have revealed how various receptors bind differently to structurally similar canyons found on the surface of virus particles (Fig. 1) (reviewed in Rossmann *et al*., 2002).

ICAM-1, PVR, and CAR are membrane-anchored glycoproteins that belong to the Immunoglobulin (Ig) superfamily. Their extracellular regions comprise five, three, and two domains, respectively, each with Ig-like folds consisting of a β barrel in which all β strands run parallel or antiparallel to the long axis of the domain (Fig. 1). In the three cases, the amino-terminal domain D1 contains the virus recognition site. This may reflect the steric capacity of the N-terminal Ig domain to penetrate into the picornaviral canyon. All the various receptor molecules utilized by picornaviruses are long, thin, and articulated at hinges between domains, and their properties are consistent with the requirement that the receptor be a molecule able to flex sufficiently to recognize additional sites on the viral surface once the first receptor site has been bound.

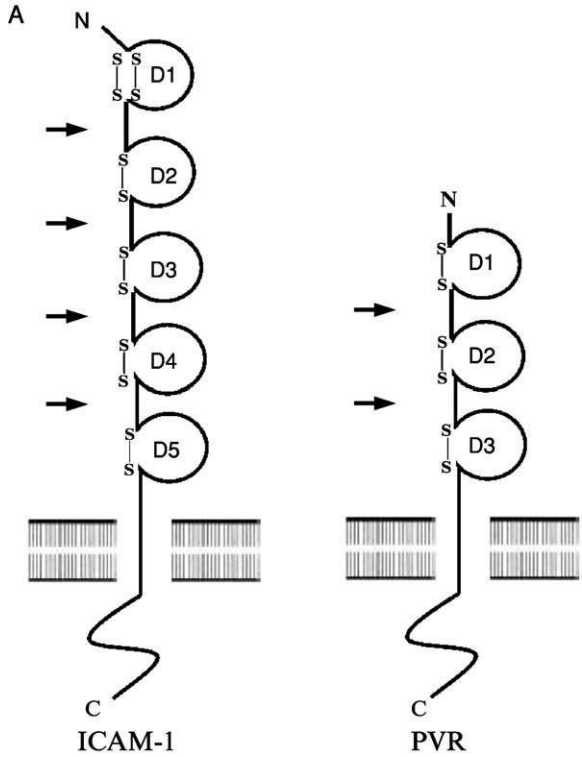
The interaction of ICAM-1 with the major group HRVs and with CAV21 and of PVR and CAR with PV1 and CBV3 indicates a common core of partially conserved residues on the canyon of those viruses (He *et al*., 2001; Rossmann *et al*., 2000; Xiao *et al*., 2001). However, the orientation of the receptor molecules is different for each virus–receptor complex (Fig. 1). The orientation of domain D1 is approximately radial in all cases except for poliovirus. The canyon of poliovirus is wider than that of CBVs and HRVs, allowing the tangential binding of PVR into the PV canyon (Belnap *et al*., 2000;

He *et al*., 2000; Xing *et al*., 2000). Therefore, the shape and size of the canyon may be important factors that dictate the docking orientation of the receptors. Receptor binding to major group HRVs and enteroviruses is localized within the canyon, at a site adjacent to a hydrophobic pocket within the VP1 β barrel containing an as yet unidentified "pocket factor." Kinetic analyses of the virus–receptor interaction have shown for both HRVs (Casasnovas and Springer, 1995) and polioviruses (McDermott *et al*., 2000) that there are two distinct modes of binding whose relative abundance varies with temperature. The binding modes observed in the cryo-EM reconstructions are likely to be the most stable intermediates, although the nature of the interaction may depend on the specific virus–receptor complex.

In contrast to observations with the major group rhinoviruses, cryo-EM reconstructions of the complex between HRV2 and the first three ligand-binding repeats of the very low-density lipoprotein (VLDL) receptor revealed that the receptor for minor group rhinoviruses binds to a star-shaped dome on the five-fold axis and not in the canyon (Hewat *et al*., 2000). The footprint of V1-3 includes only residues of VP1. Close to the receptor attachment site is the virus-neutralizing immunogenic site A, located within the BC loop of VP1. Because the receptor-binding site of HRV2 is on a protruding domain of the capsid, it is not hidden from immune surveillance. It is remarkable that the receptor-binding site of major group HRVs is very similar to that of enteroviruses, which belong to a different genus, but is essentially different from that of minor group HRVs, which belong to the same genus. Minor group HRVs, which do not bind ICAM-1, are not obviously phylogenetically or structurally distinct from major group HRVs. Furthermore, HRV14, a major group serotype, is more distantly related to another major group serotype, HRV16, than to the minor group representative HRV2. Nevertheless, the residues of HRV2 corresponding to the ICAM-1 footprint on HRV14 or HRV16 lack the charge complementarity observed for major group HRVs (Kolatkar *et al*., 1999; Verdaguer *et al*., 2000). In addition, receptors of major group HRVs and enteroviruses both cause virus destabilization, unlike the receptor of minor group HRVs. This is an example of the adaptability to different receptors and the variety of receptor-binding sites exhibited by members of the picornavirus family. A comparison of receptor interactions and entry pathways of different picornaviruses suggests that those receptors that bind into canyons or pits of the capsid induce partial capsid destabilization through displacement of the "pocket factor," which contributes to capsid stability. This type of receptor-mediated destabilization occurs in poliovirus, some coxsackieviruses, and the

major group rhinoviruses, which utilize molecules of the Ig superfamily as receptors. In contrast, those receptor molecules that do not bind into the canyon often do not contribute to virus destabilization and may condition the nature of subsequent steps in the entry process (a destabilizing, acid-mediated step in endosomes, etc.). Examples include the binding of VLDV receptor to the five-fold axis region of the minor group of HRV-2, echovirus 7 binding to DAF and FMDV binding to integrins or other receptor molecules (Table III). It has been suggested that viruses that do not use molecules of the Ig superfamily as receptors may be more variable at receptor recognition sites, facilitating shifts in receptor usage, with consequences for virus pathogenesis (Rossmann *et al*., 2002).

Other cryo-EM studies have provided indirect evidence about the location of the receptor-binding sites of reoviruses, alphaviruses, adenoviruses, and the picornavirus FMDV. For example, studies on the structure of VP4, the 88-kDa receptor recognition protein of rotavirus,



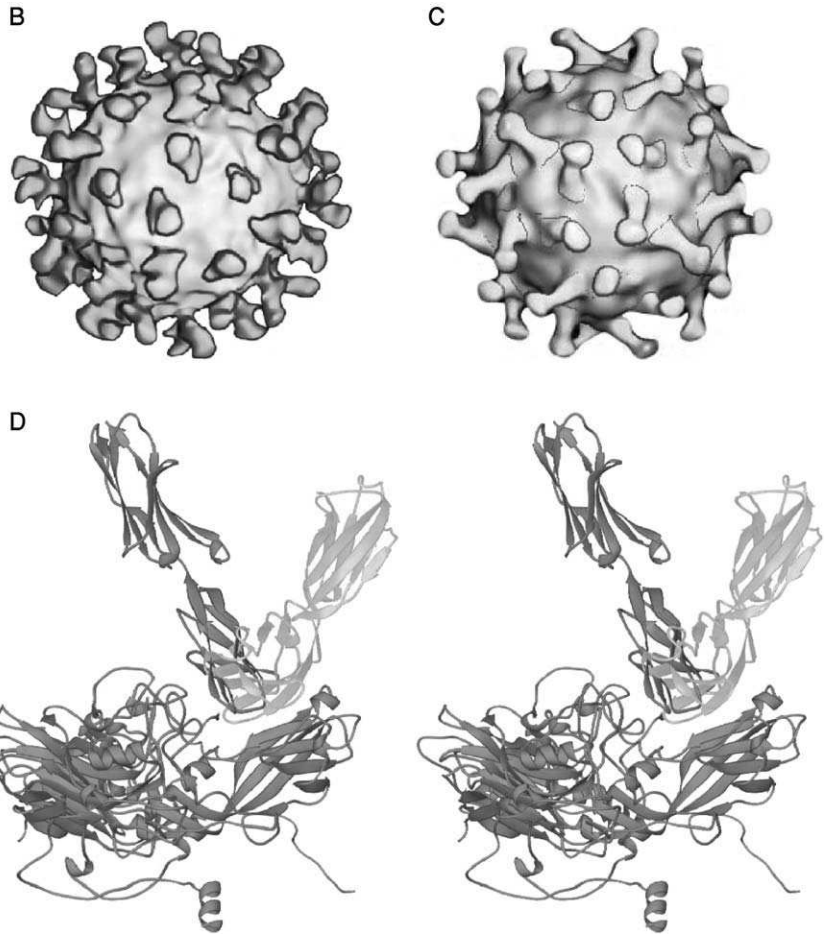


FIG 1. (A) Representation and comparison of the domain structure of ICAM-1 (CD54), the receptor for the major group rhinoviruses, and the poliovirus receptor (PVR or CD155). The immunoglobulin-like domains (labeled D1–D5 or D1–D3) are represented schematically by a circle closed by one or two disulfide bonds. The different Ig domains are linked by a flexible peptide chain. Hinge points are indicated by arrows. (B) Cryo-EM reconstruction showing the complex of HRV16 with its ICAM-1 receptor (from Kolatkar *et al*., 1999) virus is represented as a gray-scale surface. D1 and D2 domains of ICAM-1 are colored red. (C) Cryo-EM reconstruction of the complex of PV1(M) (gray) with PVR (yellow) (from Xing *et al*., 2000). (D) ICAM-1 and PVR-binding modes. Stereoview of the ICAM-1 (red) docked onto one icosahedral asymmetric unit of HRV16 (gray) using the cryo-EM map as a guide (PDB accession code 1D3E). The structure of PVR in complex with PV1 (M) (PDB accession code 1DGI) was superimposed for comparison (yellow). ICAM-1 contacts primarily the floor and south wall of the HRV16 canyon. In contrast, PVR overlaps the north and south walls, as well as the floor of the canyon, making additional contacts with the viral surface. (See Color Insert.)

have documented that 60 dimers of VP4 ("spikes") extend 100 Å above the viral capsid surface in a uniform arrangement that appears to facilitate the binding interaction with cellular receptors (Prasad *et al.*, 1988, 1990). The distal ends of the spikes are believed to contain the receptor-binding sites, as neutralizing antibodies that bind near the distal ends inhibit viral penetration (Prasad *et al.*, 1990). Cryo-EM and three-dimensional reconstructions have been valuable tools for identifying putative receptor-binding sites on the E2 glycoproteins of two alphaviruses, Sindbis virus and Ross River virus (Smith *et al.*, 1995). This strategy has also facilitated the examination of a highly mobile, Arg-Gly-Asp (RGD)-containing antigenic loop on the adenovirus 2 and 12 particles (Stewart *et al.*, 1997; Chiu *et al.*, 1999) and on the picornavirus FMDV capsid (Hewat *et al.*, 1997; Verdaguer *et al.*, 1999), which harbor integrin-binding sites (Fig. 2).

Detailed understanding of virus–receptor interactions will ultimately require structural analyses at high resolution. Crystallographic studies of interactions between FMDV O1 and HS have shown that the binding site is a shallow depression, of positive electrostatic charge, on the virion surface, contributed by the three external capsid

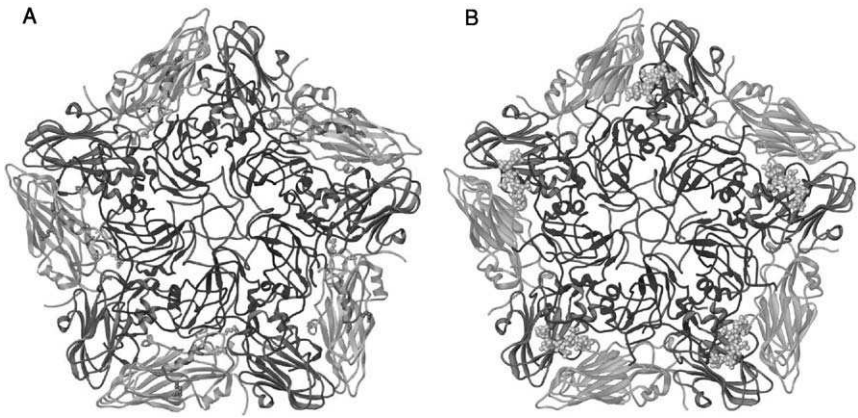


FIG 2. (A) Ribbon representation of a CS8c1 pentamer subunit (VP1, blue; VP2, green; VP3, red). The mobile antigenic G-H loop of VP1 (residues 130–160) is highlighted in yellow in a position corresponding to that found in the complex with the neutralizing antibody SD6 (Hewat *et al.*, 1997) and in cyan for the position determined in the crystallographic structure of the reduced FMDV-O1BFS (Logan *et al.*, 1993). The RGD integrin-binding triplet is depicted as sticks. (B) The structure of FMDV in complex with heparin (Fry *et al.*, 1999). Heparin coordinates for five sugars are shown as yellow ball and sticks. (See Color Insert.)

proteins VP1, VP2, and VP3 (Fry *et al*., 1999) (Fig. 2). The crystal structure revealed that the heparin molecule makes ionic interactions with the viral residues using two sulfates (GlcN-2-2-*N*-SO_3 and GlcN-4-6-*O*-SO_3). Amino acid Arg-56 in VP3 plays a central role in organizing the sulfate groups, making interactions with both. Further polar residues that belong to all three major capsid proteins play a subsidiary role in heparin binding via bridging water molecules. The nonionic interactions observed include van der Waals stacking contact among, His-195 of VP1, the central L-iduronic acid (Idu) ring, and the apolar patch of GlcN-4. The virus surface remained essentially unchanged to accommodate the sugar, suggesting that the shape complementarity may contribute to receptor specificity.

Crystallographic studies of interactions between the HIV gp120 in complex with its receptors (Kwong *et al*., 1998; Wyatt *et al*., 1998) and the structurally unrelated adenovirus 12 knob with CAR (Bewley *et al*., 1999) allowed the identification of key determinants of receptor-binding specificity. These structures revealed that the receptor-binding faces are surface loops exposed to immune selective pressure. Both viruses bury a similar amount of surface area to create an atypical virus–receptor interface because of the presence of a shape mismatch in the surface topography that creates large cavities or channels. The lack of van der Waals interactions between the two protein surfaces may be partially compensated by the presence of solvent molecules filling the cavities. These water molecules would act as a bridge mediating hydrogen bonds between the backbone atoms of the viral protein and the receptor. The viral residues in contact with this water-filled cavity show important sequence variability, whereas surrounding this patch are highly conserved residues, the substitution of which may affect receptor binding. Thus, the observed interfacial cavities may serve a dual purpose as a water buffer between the viral protein and the receptor, and as molecular glue through the establishment of hydrogen bonds between backbone and conserved atoms. The tolerance for variation in the surface of the protein associated with this cavity produces a variational island, which is centrally located between regions required for receptor binding and may help the virus escape from antibodies. By using a noncomplementary interface that traps water molecules, the virus can maintain its receptor specificity while altering its amino acid sequence. This type of interface observed in two very different viruses such as HIV and adenovirus supports the water buffer hypothesis as a new general mechanism by which viruses can complete the first stage

of infection successfully. The trapping of water molecules may allow receptor recognition to be less influenced by amino acid substitutions at the relevant sites of the virus surface, minimizing the adverse effects of high mutational pressure (Section II,B) on an essential step in the virus life cycle.

IV. Quasispecies and Shifts in Receptor Usage

A. Minimal Changes in Viral Genomes May Modify Receptor Recognition or Cell Tropism

There are several cases of minimal changes in viral genomes that result in the alteration of receptor specificity or affinity (Baranowski *et al.*, 2001). Early studies with influenza virus identified key residues of virus hemagglutinin (HA) that confer specificity for sialic acid linked to galactose by either an α-2,3 or an α-2,6 linkage. Human H3 influenza viruses bind preferentially to sialic acid linked to galactose by an α-2,6 linkage, whereas avian and equine viruses show a preference for the α-2,3 linkage (Matrosovich *et al.*, 1997; Rogers and Paulson, 1983). Human H1 and H2 viruses, as well as swine H1 viruses, also show preferentially a specificity for Neu5-Acα-2,3Gal. It was established that a single substitution in the 226 position of the HA changed the receptor specificity, which suggests that HAs that differ in the recognition of one sialic acid or another differ in amino acid 226 (Rogers *et al.*, 1983a). Further studies indicated that two mutations in residues 226 and 228 of a human HA allowed replication in ducks. The mutations resulted in a receptor-binding site sequence identical to the known avian influenza virus sequences (Naeve *et al.*, 1984). Other studies on receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates showed that residues 226 and 228 are leucine and serine in human isolates and are glutamine and glycine in avian and equine isolates (Connor *et al.*, 1994), confirming the key role of these two residues in determining the receptor specificity of influenza viruses.

Picornaviruses provide additional examples. Single replacements (Val-160 to Ile in VP1 or Met-62 to Ile in VP4) allow the primate-restricted P1/Mahoney strain of poliovirus to paralyze mice (Colston and Racaniello, 1995). The mutation in VP4 may render the virus accessible to a molecule that acts as a virus receptor and which is located on the surface of neurons of the mouse spinal cord. This molecule does not seem to be expressed in the mouse brain (Jia *et al.*, 1999). A mutant

of encephalomyocarditis virus termed variant D is usually asymptomatic for rodents, but it can induce diabetes in mice through the destruction of pancreatic β cells. The diabetogenic variant includes amino acid replacement Thr-776 to Ala along the capsid pit, likely affecting receptor interaction and cell tropism (Bae and Yoon, 1993). Important determinants of tropism and pathogenesis of Theiler's murine encephalomyelitis virus (TMEV) have been mapped at various positions on capsid protein VP1, including positions adjacent to the putative virus receptor-binding site. TMEV mutants harboring single amino acid substitutions at these particular VP1 positions displayed altered neurovirulence in susceptible mice and produced only mild symptoms during the acute phase of the infection or were highly attenuated regarding the development of chronic demyelinating disease (Jnaoui *et al*., 2002; Lin *et al*., 1998; McCright *et al*., 1999; Wada *et al*., 1994). Amino acid residues flanking the RGD integrin-binding motif of FMDV have been shown to influence the selectivity of integrin binding (Jackson *et al*., 2000a, 2002).

Research on the Edmonston vaccine strain of measles virus (MV) led to the identification of CD46 (a group of determinants whose function is to protect cells from complement-mediated lysis) as a receptor for this virus. Transgenic mice expressing CD46 may show typical pathogenic manifestations of the virus (Oldstone *et al*., 1999). Marmosets lacking CD46 were susceptible to several isolates of MV, but not to the Edmonston vaccine strain. Some natural isolates of MV do not enter cells through CD46, instead they use the signaling lymphocyte activation molecule (SLAM or CDW150), a glycoprotein expressed on some types of B and T lymphocytes, as a receptor (Hsu *et al*., 2001; Tatsuo *et al*., 2000a, 2000b). Although wild-type MV interacts with SLAM with high affinity, it can also interact with CD46 with low affinity (Masse *et al*., 2002). A single amino acid replacement in the surface hemagglutinin of the MV envelope determines the ability of the virus to bind CD46 with high affinity (Hsu *et al*., 1998). Additional receptors may be involved in MV infection (Hashimoto *et al*., 2002; Oldstone *et al*., 2002). Despite its relative antigenic stability, it has been estimated that MV mutates at an average rate of 9×10^{-5} substitutions per base copied (Schrag *et al*., 1999). Therefore, point mutations of the type that led to binding of CD46 should occur frequently during MV replication.

Another example of minimal change in a viral genome that affects receptor recognition, tropism, and pathogenesis is afforded by substitutions in the E2 surface glycoprotein of Sindbis virus. The presence of Arg at position 172 impaired neurovirulence through a decrease of

binding to a receptor on neural cells (Tucker and Griffin, 1991); the opposite occurred when position 172 was a Gly. Also, position 55 of E2 is critical for binding to neurons under certain environmental conditions (in media with a similar ionic strength and degree of sulfation than found in interstitial fluid). While Gln-55 increases binding to neurons, His-55 (a substitution that entails a single nucleotide mutation) stabilizes the interaction between Sindbis virus and the surface of neural cells, contributing to a greater neurovirulence (Lee *et al*., 2002).

Studies on HIV coreceptor usage in cell culture revealed that the presence of either Lys or Arg at position 306, or Lys at position 322 within the variable V3 loop of gp160, led to a switch from using CCR5 to CXCR4 to enter cells (De Jong *et al*., 1992; Fouchier *et al*., 1995). *In vivo*, the change to using the CXCR4 coreceptor was also linked to a number of mutations that implied an acquisition of positive charges and loss of a N-glycosylation site, modifications that clustered between amino acids 190 and 204 og gp160. In other cases, substitutions to nonbasic amino acids at position 440 of gp160 were also linked to the R5 or X4 phenotypes (Hoffman et al., 2002). Furthermore, changes that confer a highly basic character (on loop V3) allow the virus to also use glycosaminoglycans as attachment factors for cell entry, which results in a 10-fold increase in viral production in cell culture (Zhang *et al*., 2002).

The cellular tropism of another lentivirus, feline immunodeficiency virus (FIV), may be affected by mutations in the V3 region of the surface glycoprotein SU. A change of cell tropism in cell culture was associated with replacement Glu-407 to Lys (Verschoor *et al*., 1995). Crandell feline kidney (CRFK) cells were transfected with an FIV molecular clone that was unable to infect them. However, high-dose DNA transfection resulted in the recovery of an FIV mutant capable of replicating in these cells. A single point mutation in the SU protein was responsible for this change in viral tropism (Vahlenkamp *et al*., 1997). In another study, a variant of FIV emerged after the passage of wild-type virus in cell culture. The variant displayed a phenotype markedly different from that of the parental virus, including the capacity to productively infect previously refractory cell lines and the induction of large syncytia. This phenotype could be attributed to the combination of two amino acid substitutions (Gln-224 to Pro, Thr-470 to Pro) in glycoprotein SU and to a premature stop codon that resulted in a truncated transmembrane protein (Lerner and Elder, 2000).

Cases of amino acid replacements affecting cell recognition have also been described in small and complex DNA viruses. Modifications of the

host range of parvoviruses have been associated with amino acid substitutions on the viral capsid (Parrish and Truyen, 1999). Binding affinity to the transferrin receptor correlated only partially with the host range displayed by the virus (Hueffer *et al*., 2003). Remarkable changes in adenovirus (Ad) host cell tropism can be observed upon minimal alterations of virus fiber sequences. Amino acid substitution Glu-240 to Lys in the distal domain of Ad19p fiber conferred binding to human conjunctival cells, whereas the reverse substitution abrogated cell binding when introduced into the phylogenetically distant Ad37 (Huang *et al*., 1999). The presence of a Lys residue at position 240 of the adenovirus fiber was associated with an outbreak of epidemic keratoconjunctivitis.

Single amino acid substitutions in glycoprotein gD of herpes simplex 1 (HSV-1) can alter receptor preferences (reviewed in Spear *et al*., 2000). Although wild-type HSV-1 strains can enter cells via HveA (but not via human nectin-2), Rid mutants, which differ from the wild type by an amino acid substitution at position 25 or 27 of gD, can use human nectin-2 (but not human HveA). Studies with the swine alphaherpesvirus pseudorabies virus (PrV) further documented the important potential of herpesviruses to explore alternative mechanisms of cell recognition (Nixdorf *et al*., 1999; Schmidt *et al*., 1997, 2001). Although glycoprotein gD is critically involved in PrV entry into susceptible cells, the expression of gD is not required for direct viral cell to cell spread and PrV mutants deleted in the gD gene can be propagated in MDBK cells by cocultivating infected and noninfected cells. While infectivity was found to be strictly cell associated in early passages, repeated passaging resulted in the appearance of infectivity in the supernatant, reaching titers as high as 10^7 PFU/ml. Mutations in viral glycoproteins gB and gH were found to correlate with the capacity of PrV to interact with alternative receptors and the development of a gD-independent mode of entry.

This by no means exhaustive list of examples underscores the biological relevance for RNA viruses of quasispecies dynamics regarding alterations in host cell tropism and pathogenic manifestations of viral infections. The weight of single nucleotide substitutions in the phenotype of RNA viruses must be considered in relation to the additional three parameters indicated in Table I (number of mutations per genome, virus population size, and genome length). The fact that a single replacement can have profound biological consequences for an RNA virus was one of the triggers of interest in quasispecies (review of early observations in Domingo *et al*., 1985). To what extent complex DNA viruses may incorporate mutations at some genomic sites

involved in cell recognition, with the flexibility of RNA viruses, is largely unknown. Because of several implications for the biology and uses of DNA viruses (discussed in Section V), this problem certainly deserves further investigation (compare with Section II,B).

B. Changes in Receptor Specificity upon Virus Evolution in Cell Culture and in Vivo

A modification of receptor specificity of FMDV was observed on the long-term passage of FMDV C-S8c1 [a biological clone derived from natural isolate C-Sta Pau Sp/70, representative of serotype C FMDV (Sobrino *et al*., 1983)] in BHK-21 cells. The parental clone C-S8c1 enters BHK-21 cells via an RGD-dependent integrin, as documented by both inhibition of cell entry by synthetic peptides and site-directed mutagenesis of an infectious cDNA clone to modify the RGD (Baranowski *et al*., 2000). Passage of FMDV C-S8c1 in BHK-21 cells resulted in the dominance of viruses with several amino acid replacements on the capsid surface (Fig. 3). These replacements resulted in an expansion of host cell tropism in cell culture, with the acquired capacity to infect a number of primate and human cell lines, a capacity that was absent in the parental C-S8c1 (Baranowski *et al*., 2000; Ruíz-Jarabo *et al*., 2002). As expected, some of the amino acid replacements conferred to the multiply passaged virus the capacity to bind heparin.

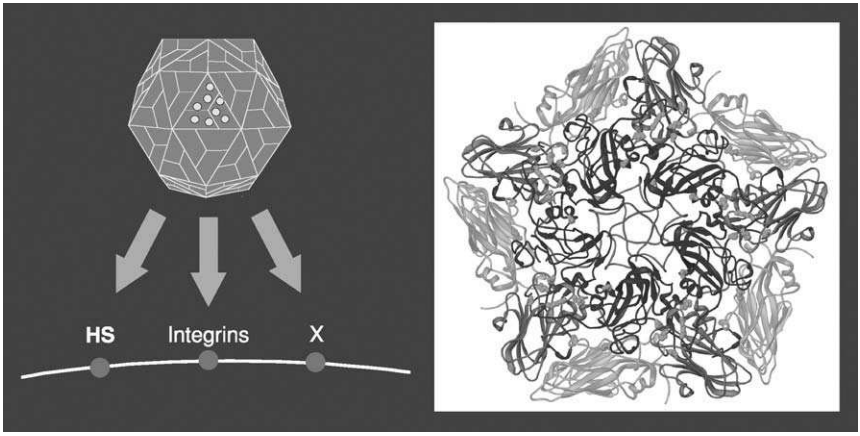


FIG 3. Flexibility in receptor usage by FMDV. Passage of FMDV in BHK-21 cells resulted in acquisition of amino acid replacements in the capsid, which expanded receptor usage (HS, heparan sulfate; integrin $\alpha_v\beta_3$; X, an unidentified receptor). Replacements are depicted as Van der Waals spheres in yellow (VP1, blue; VP2 green; VP3, red). (See Color Insert.)

However, these viruses did not need to use HS as a receptor, as mutants deficient in heparin binding (that were selected from the multiply passaged quasispecies) were equally infectious in cell culture (Baranowski *et al*., 2000). Therefore, a third entry pathway, which is independent of HS and of RGD-dependent integrins, must have been acquired by FMDV C-S8c1 upon repeated passage in BHK-21 cells. The selective changes underlying such cell recognition conversion are not known (Baranowski *et al*., 2000, 2001).

The acquisition of a third entry pathway by FMDV did not abrogate a potential use of RGD-dependent integrins. Indeed, using the mutants from the multiply passaged FMDV C-S8c1 that were deficient in heparin binding, it was documented that RGD containing peptides (but not the same peptides with RGG instead of RGD) inhibited the infection of BHK-21 cells (Baranowski *et al*., 2000). Therefore, when HS binding was impaired, the virus used the RGD-dependent pathway for cell entry. These results prove that modifications in receptor recognition can be produced readily by modest evolutionary transitions and that a virus can maintain the capacity to penetrate the same cell type via three alternative entry pathways (Baranowski *et al*., 2000, 2001) (Fig. 3).

Some lines of evidence suggest that the use of alternative receptors for FMDV also occurs *in vivo*. Variants of FMDV harboring replacements within the RGD or at some neighboring positions documented to be critical for integrin binding have been isolated *in vivo*. One of the relevant experiments involved vaccination of 138 cattle with synthetic peptides representing B-cell and T-cell epitopes of FMDV C3 ARg-85 (Taboga *et al*., 1997; Tami *et al*., 2003). The animals were protected only partially as a result of the immune response to the synthetic peptide constructs and, upon challenge with virulent FMDV C3 Arg-85, several of them developed viremia and vesicular lesions. Virus from several lesions included amino acid substitutions within the RGD or at neighboring positions (Fig. 4). It is not clear whether such variant viruses would be able to reinitiate infection of cattle and, if they did, whether they would be stable through the completion of infectious cycles *in vivo*. Passage of FMDV O/CHN/90 (a type O FMDV used for vaccine production in China) in BHK-21 cells resulted in viruses that displayed altered tropism. Cell culture adaptation of the Cathay prototype foot-and-mouth disease virus from China results in altered tissue culture host range and pathogenic phenotype in pigs (Zhao *et al*., 2003). This altered virus caused mild disease in swine. Despite this evidence for flexibility in receptor usage, variants of FMDV selected to bind heparan sulfate were attenuated in

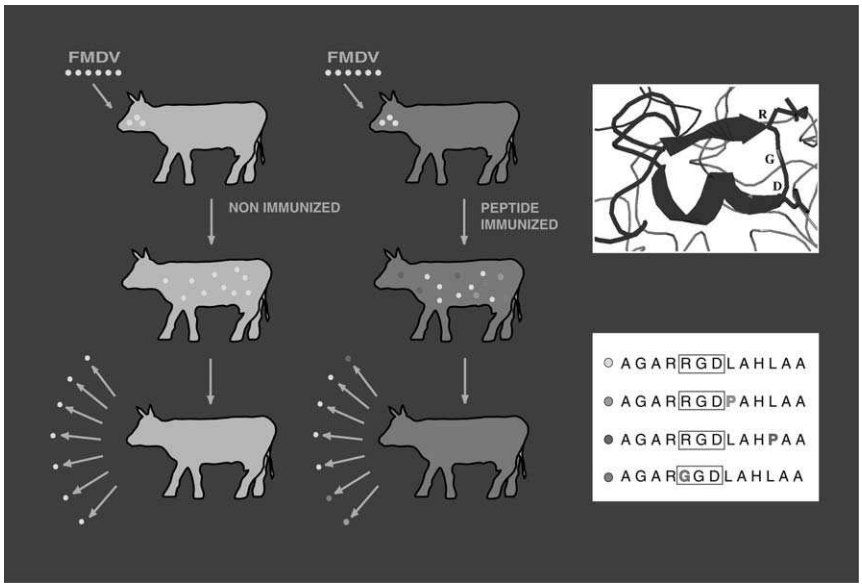


FIG 4. Selection of FMDV variants in peptide-immunized cattle. In lesions from the immunized animals challenged by a cloned virus, variants with amino acid replacements within or near the RGD integrin receptor-binding triplet were isolated. The position of the RGD in an open turn between a β strand and an helical region of VP1 is indicated in the box (VP1, blue; VP2, green; VP3, red) above the sequence alignment, indicating the amino acid replacements. (See Color Insert.)

cattle (Sa-Carvalho *et al.*, 1997). This and other observations suggest that RGD-dependent integrins may be a major class of receptors employed by FMDV *in vivo* (Neff *et al.*, 1998), although the flexibility of the virus to modify receptor usage *in vivo* is an open question.

Variants of the arenavirus lymphocytic choriomeningitis virus (LCMV) arise during long-term persistent infections in mice (Ahmed *et al.*, 1984; reviewed in Sevilla *et al.*, 2002). Some of the variants had different biological properties, expected from the tissues from which they were isolated. One type of variant predominated in the central nervous system (CNS), whereas another type was dominant in lymphocytes and macrophages. Most CNS isolates caused acute infection when injected intravenously into immunocompetent adult mice. These variants evoked a potent immune response and infection was cleared with the active participation of anti-LCMV $CD8^+$ cytotoxic T lymphocytes (CTLs). In contrast, most LCMV variants from lymphocytes and macrophages evoked a generalized immunosupression in the animals. Interestingly, these critically different phenotypes

of LCMV relate to alterations in the affinity for α-dystroglycan, a receptor for LCMV, lassa fever, and clade C new world arenaviruses (Cao *et al.*, 1998; Spiropoulou *et al.*, 2002). A single amino acid substitution in the glycoprotein GP1 of LCMV alters its affinity for α-dystroglycan; high-affinity binding is associated with immunosupression and viral persistence in mice, whereas low-affinity binding results in clearence of infection (Cao *et al.*, 1998; Sevilla *et al.*, 2000, 2002; Smelt *et al.*, 2001).

HIV-1 coreceptor usage changes during infection in humans. Most primary isolates belong to the coreceptor specificity R5, but as infection progresses, dualtropic (R5X4) and X4 variants arise and often become dominant (Section III,A). The emergence of X4 HIV-1 strains in infected individuals has been associated with an increase in pathogenic manifestations and development of AIDS. Evolution of coreceptor usage (R5, multitropic, X4) seems to be the rule with HIV-1 subtypes A, B, D, and E during progressive disease. Subtype C apparently does not follow this trend in that the great majority of isolates obtained at different geographic sites are R5 monotropic regardless of the severity of the HIV infection (Fenÿo, 2001). There are variants proven to be able to use other coreceptors as well, such as CCR2b, CCR3, CCR8, BOB, Bonzo, US28, and BLTR (B_4 leukotriene receptor), but their relevance for HIV infection *in vivo* is not clear (Fenÿo, 2001). The host range of HIV-2 in human cells is similar to that of HIV-1, yet differences exist in the use of coreceptors. Primary isolates of HIV-2 use CCR5, but they may simultaneously use a wide variety of other coreceptors, such as CCR1, CCR2b, CCR3, CCR5, and BOB (Table III), despite the main coreceptor being CCR5. Variants that can use CXCR4 become dominant at late stages of the infection (Morner *et al.*, 1999). Multitropic HIV-2 and SIV strains can infect CD4-negative cells (Liu *et al.*, 2000). SIVsm is closer phylogenetically to HIV-2 than to HIV-1 and behaves similarly in several ways. As HIV-2, SIVsm can use a wide variety of different coreceptors, such as CCR3, CCR5, CXCR4, BOB, and Bonzo. They are multitropic, and several isolates can infect CD4-negative cells (this is also true for a small number of HIV-1 strains). Coreceptor usage, in contrast to what it is observed with HIV-1, narrows with time, and as the infection progresses, HIV-2 isolates tend to use only coreceptor CXCR4, and SIV only CCR5 (Vodros *et al.*, 2001).

Feline leukemia virus A (FeLV-A) is the form of FeLV that is transmitted from cat to cat, and for this reason it is called the ecotropic form. It does not cause acute disease and is restricted to grow on feline cells. The receptor for this group of viruses has not been isolated. FeLV-Bs evolve from FeLV-A by recombination with endogenous FeLV-like

(enFeLV) envelope sequences, which results in a change to Pit1 receptor specificity. Pit1 is a classic multiple membrane-spanning receptor molecule. Recombinant forms of FeLV-B differ in the amount of envelope surface unit (SU) that is derived from enFeLV, and this may affect whether the virus can also use Pit2 (which has 62% structural identity with Pit1) as a receptor (Anderson *et al.*, 2001; Sommerfelt, 1999; Sugai *et al.*, 2001; Tailor *et al.*, 1999b). Viruses that use Pit1 and Pit2 as receptors use the latter with lower efficiency (Boomer *et al.*, 1997). FeLV-C and -T evolve by mutation of FeLV-A. In contrast to group A, groups B, C, and T FeLVs show an expanded host range, which includes infection of human cells (Sommerfelt, 1999; Sugai *et al.*, 2001). FeLV-C viruses use a protein called feline leukemia virus subgroup C receptor (FLVCR), and the use of this receptor is associated with amino acid replacements within the 15 to 20 amino acids of the first variable region (V1) of SU. FeLV-C strains differ from FeLV-A in a Lys-to-Arg substitution in V1, but additional substitutions in SU must be involved in the use of FLVCR and the expanded host range (Brojatsch *et al.*, 1992; Sommerfelt, 1999). FeLV-T (a T-cell tropic feline leukemia virus) is the first naturally occurring type C retrovirus that cannot infect cells unless both Pit1 and a second coreceptor or entry factor are present. This second receptor component, called FeLIX, is a cellular protein that is closely related to a portion of the FeLV envelope protein. This cellular protein can function either as a transmembrane protein or as a soluble component to facilitate infection (Anderson *et al.*, 2000; Lauring *et al.*, 2001). The most important feature to confer T-cell tropism seems to be an insertion of four to six amino acids in the C-terminal portion of SU, although additional amino acids could also be involved in this phenotype.

Friend virus is a nonneuropathogenic ecotropic murine leukemia virus. Variant PVC-211 isolated after 30 passages of Friend virus in BALB/c mice caused a rapidly progressive hind limb paralysis when injected into newborn rats and mice and displayed an expanded tropism in tissue culture. The two mutations responsible for the altered phenotype were Glu-116 to Gly and Glu-129 to Lys in the surface protein of the virus (Kai and Furuta, 1984; Masuda *et al.*, 1992, 1996a, 1996b).

C. Nature of the Selective Forces That Drive the Selection of Virus Variants

It is very difficult to identify the selective pressures that act on viruses during an infectious process, not only because of the complexity of host influences involved, but also because selective pressures may vary

due to physiological alterations that often accompany the infection. HIV infections, with their erosive effects on the immune system, offer a relevant case. Adaptation of HIV to a changing cellular environment could contribute to the selection of variants with different cell tropism and different pathogenicity (Viscidi, 1999). This can be regarded as a positive selection directed by the immune system, resulting in modifications of host cell tropism of HIV (Crandall, 1999). For example, in the late stages of HIV infection, $CD4^+$ cells are depleted, and variants that use CD8 to enter $CD8^+$ cells arise (Saha *et al*., 2001). Thus, subpopulations of cells involved in the human immune response, whose abundance may be modified by HIV-1 infection, may in turn become a key selective force for HIV evolution (Crandall, 1999).

The relative availability of chemokine receptor ligands may also be a selective pressure involved in HIV evolution *in vivo*. Stromal-derived factor 1 (SDF-1), a chemokine that is a CXCR4 ligand, is expressed constitutively by mucosal epithelial cells at sites of HIV transmission and replication (Agace *et al*., 2000), and this may be one of the factors implicated in the selective transmission of R5 HIV-1 strains. Individuals whose peripheral blood lymphocytes produce high levels of CCR5 ligands (such as RANTES, MIP1α, and MIP1β) are relatively resistant to infection (Garzino-Demo *et al*., 1998; Paxton *et al*., 1996; Zagury *et al*., 1998). Selection by receptor ligands is also supported by experiments using a mouse model in which a modified form of RANTES, a natural ligand for CCR5, selected HIV mutants that used CXCR4 as a coreceptor (Mosier *et al*., 1999). Also, AMD3100, a bicyclam that is a selective antagonist of CXCR4, led to the complete suppression of X4 variants in cell culture and prevented the switch from the less pathogenic R5 HIV to the more pathogenic X4 strains (Este *et al*., 1999).

Multiplication in a given type of cell may also be a likely trigger of a tropism alteration. Measles virus isolated on marmoset B-cell lines infected some primate B- and T-cell lines and retained pathogenicity for monkeys. This was not the case for measles virus isolated on Vero cells, as such isolates manifested a different tropism and host range (Tatsuo *et al*., 2000a, 2000b), suggesting that the type of cell used for measles virus isolation exerted a selective force on the virus. Likewise, the host cell selected an avian retrovirus variant with an expanded host range, consisting of recognition of a receptor on chicken cells and a distinct receptor on quail cells (Taplitz and Coffin, 1997). This evidence must make virologists aware that when they isolate a virus from a biological specimen by passage in an established cell line, a virus with different biological properties (cell tropism or other) than

the natural virus may be selected. Variants of LCMV with profoundly different biological properties have been isolated from different organs of infected mice (Section IV,B), emphasizing the impact of quasispecies dynamics during infections *in vivo* (Sevilla *et al.*, 2002). In most cases summarized in this section, the precise nature of the selective force that acts to perturb mutant distribution is not well understood in molecular terms. In many other cases, even the types of selective constraints that mediate changes in host cell tropism are not obvious. This is perhaps one of the areas of research in evolutionary virology that necessitates efforts because of the multiple implications of variations of host cell tropism for the control of viral disease. Model experiments in cultured cells (that attempt to match the type of cells found *in vivo*), with the aim of relating variations in the nucleotide sequence (and in the corresponding structure of encoded proteins) with alteration in cell recognition, may provide information on the molecular basis for tropism specificity and tropism changes.

V. Biological Implications of Modifications in Receptor Usage

A. Coevolution of Receptor Usage and Antigenicity

Structural studies have shown in many cases that there is an overlap between the amino acid residues on a viral capsid or envelope that are involved in cell recognition and those that recognize neutralizing antibodies (Section III,C) (Table IV). The structure of the complex between the G-H loop of VP1 of FMDV with monoclonal antibody 4C4 has clear resemblance with the complex that can be modeled between the virus and its integrin receptor (Fig. 5). A change in receptor specificity could involve a modification in binding to antibodies or vice versa. Furthermore, an antigenic domain that coincides with a receptor-binding site may lose constraints for variation once a different receptor that interacts with other capsid residues becomes operational. The latter possibility can be illustrated with results obtained with FMDV.

The capacity of FMDV to use multiple alternative receptors for entry even into the same cell type (Section IV,C) confers to this virus the potential to modulate receptor usage in response to selective constraints (Baranowski *et al.*, 2000), which has considerable implications for the evolution of virus antigenicity. Because the RGD triplet located at the surface G-H loop of capsid protein VP1 is a key part of several epitopes recognized by neutralizing antibodies (Mateu, 1995; Verdaguer *et al.*, 1995), dispensability of the RGD integrin-binding

TABLE IV

EXAMPLES OF OVERLAP BETWEEN ANTIBODY AND RECEPTOR-BINDING SITES IN VIRUSES

Viral system	Main observations
Adenoviridae	
Adenovirus	Synthetic peptides representing fiber knob of Ad-3 present cell receptor-binding sites and antigenic epitopes (Liebermann *et al*., 1998)
Coronaviridae	
Murine coronavirus	Mab recognized epitopes involved in the binding of virions to cellular receptors (Kubo *et al*., 1993, 1994)
Flaviviridae	
BVDV	Anti-idiotypic antibodies mimicking viral antigens bind to cellular receptors (Xue and Minocha, 1993; Minocha *et al*., 1997)
Dengue virus	Amino acid residues critical for mouse neurovirulence are involved in antibody binding (Hiramatsu *et al*., 1996)
Yellow fever virus	Amino acid residues critical for virus neurotropism are involved in antibody binding (Jennings *et al*., 1994)
Hepadnaviridae	
Duck hepatitis B virus	Residues critical for virus neutralization are involved in the interaction with cells (Tong *et al*., 1995; Li *et al*., 1996; Sunyach *et al*., 1999)
Hepatitis B virus	Anti-idiotypic antibodies mimicking viral antigens bind to cellular receptors (Petit *et al*., 1992; Hertogs *et al*., 1994; Budkowska *et al*., 1995)
	Anti-idiotypic antibodies mimicking cellular structures bind to small hepatitis B surface antigen (Neurath *et al*., 1986)
	A synthetic peptide analogue is recognized by both cell receptors and anti-HBV antibodies (Neurath *et al*., 1986)
Herpesviridae	
BHV-1	Anti-idiotypic antibodies mimicking viral antigens bind to cellular receptors (Thaker *et al*., 1994; Varthakavi and Minocha, 1996)
HCMV	Anti-idiotypic antibodies mimicking viral antigens bind to cellular receptors (Keay *et al*., 1989; Keay and Baldwin, 1991)
HSV	Anti-idiotypic antibodies mimicking viral antigens bind to cellular receptors (Huang and Campadelli-Fiume, 1996)
	Overlap between major neutralizing antigenic site and a receptor-binding domain of gD (Whitbeck *et al*., 1999)

(*continues*)

TABLE IV (*continued*)

Viral system	Main observations
Orthomyxoviridae	
Influenza virus	Amino acid residues within the sialic acid-binding pocket of virus hemagglutinin are accessible to neutralizing antibodies (Stewart and Nemerow, 1997)
	Antigenic and hemagglutinin variants selected upon egg adaptation (Robertson *et al*., 1987)
	Low-affinity neutralizing antibody response selected for receptor-binding variants of influenza virus HA (Laeeq *et al*., 1997)
	Amino acid changes at residues involved in antibody binding can modulate the hemagglutinating activity of influenza C virus (Matsuzaki *et al*., 1992)
	Passage of influenza C virus in HMV-II cells resulted in selection of antigenically distinct variants, which have an advantage in binding to the cell surface receptors (Umetsu *et al*., 1992)
	Hemagglutinin variants displayed increased resistance to neutralization (Nohinek *et al*., 1985)
	The receptor-binding specificity of the hemagglutinin can markedly influence the antigenic analysis obtained with monoclonal antibodies in HI tests (Yamada *et al*., 1984)
Picornaviridae	
FMDV	Overlap of integrin- and antibody-binding sites (Verdaguer *et al*., 1995)
	Monoclonal antibodies selected variants with altered integrin recognition (Martinez *et al*., 1997; Baranowski *et al*., 2000; Ruíz-Jarabo *et al*., 2003)
	Adaptation to cell culture may result in antigenic variation (Curry *et al*., 1996; Sa-Carvalho *et al*., 1997; Baranowski *et al*., 2000)
	Some amino acid residues involved in heparin-binding map at antigenic sites (Sa-Carvalho *et al*., 1997; Fry *et al*., 1999; Baranowski *et al*., 2000)
	Antigenic variants with altered receptor specificity can be selected *in vivo* (Taboga *et al*., 1997; Tami *et al*., 2003)
Poliovirus	Receptor recognition influenced by residues of antigenic sites (Murray *et al*., 1988; Harber *et al*., 1995)
	The exposed BC loop of capsid protein VP1 plays a critical role in receptor interactions in the mouse central nervous system (Yeates *et al*., 1991)
HRV	Neutralizing antibody to human rhinovirus 14 penetrates the receptor-binding canyon (Smith *et al*., 1996)

(*continues*)

TABLE IV (*continued*)

Viral system	Main observations
	The footprint of very low density lipoprotein receptor on HRV-2 surface covers two exposed loops of capsid protein VP1 (BC- and HI-loops) (Hewat *et al*., 2000)
TMEV	Neutralization epitopes map close to the putative receptor binding region (Sato *et al*., 1996)
	Mutations associated with adaptation to some culture cells map in antigenic sites (Jnaoui and Michiels, 1998)
Reoviridae	
Bluetongue virus	Anti-idiotypic antibodies mimicking viral antigens bind to cellular receptors (Xu *et al*., 1997)
Reovirus	Anti-idiotypic antibodies mimicking viral antigens bind to cellular receptors (Co *et al*., 1985a; Gaulton *et al*., 1985; Williams *et al*., 1988, 1989, 1991b)
Rhabdoviridae	
Rabies virus	Anti-idiotypic antibodies mimicking viral antigens bind to cellular receptors (Hanham *et al*., 1993)
	Amino acid residues critical for virus neurotropism are involved in antibody binding (Coulon *et al*., 1998)
Togaviridae	
Sindbis virus	Anti-idiotypic antibodies mimicking viral antigens bind to cellular receptors (Ubol and Griffin, 1991; Wang *et al*., 1991b; Strauss *et al*., 1994)
	Several antibodies bind to regions of the virions implicated in cell-receptor recognition (Smith *et al*., 1995)
Ross River virus	Several antibodies bind to regions of the virion implicated in cell-receptor recognition (Smith *et al*., 1995)

motif for cell entry expanded the repertoire of antigenic variants of FMDV greatly and prompted the isolation of viable antibody-escape mutants with profoundly altered antigenicity that contained mutations at the RGD triplet (Martinez *et al*., 1997; Ruiz-Jarabo *et al*., 1999). Mutants with RED, RGG, and even GGG instead of RGD were isolated. Viable viruses were obtained when the relevant mutations were engineered in an infectious clone with the sequence context of the capsid of the multiply passaged C-S8cl; RNA transcripts did not give rise to variable virus when the same mutations were engineered in the sequence context of the parental clone C-S8cl (Baranowski *et al*., 2000). Studies with FMDV containing RGG showed impaired reactivity with monoclonal antibodies specific for the relevant antigenic sites and with polyclonal antibodies raised in swine and guinea pigs using the wild-type virus as immunogen (Ruiz-Jarabo *et al*., 1999).

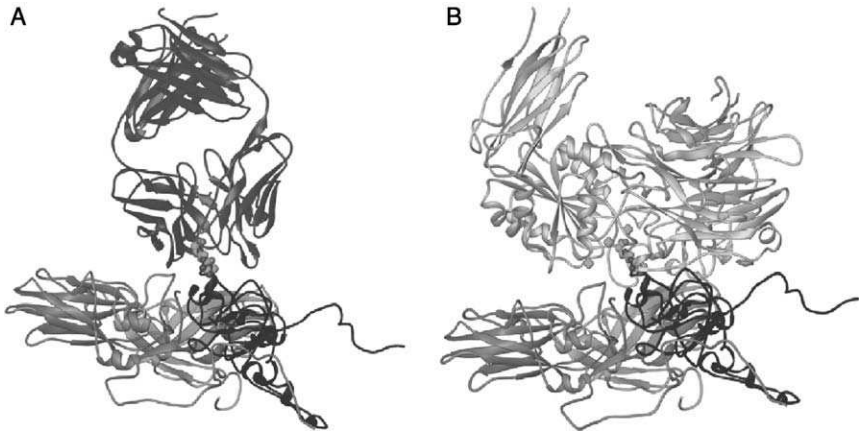


FIG 5. (A) Cryoelectron microscopy structure of the complex between FMDV and the Fab fragment from MAb 4C4 bound to the VP1 G-H loop of the virus (Verdaguer *et al*., 1999). One protomer subunit of FMDV is shown as a ribbon diagram (VP1, blue; VP2, green; VP3, red) and the Fab is in violet. The flexible G-H loop of FMDV is located in an extended orientation with the RGD motif (depicted as a Van de Waals spheres) occupying a fully exposed position. The RGD triplet, in this complex, shows a similar conformation to that found for the same triplet when bound to the integrin $\alpha v\beta 3$ (Xiong *et al*., 2002). A least-squares superimposition of the main chain atoms from RGD residues in the FMDV loop with the equivalent residues in the integrin RGD ligand gives an average rms deviation of only 0.32 Å. The transformation necessary to superimpose the FMDV loop to the integrin ligand can also be used to superimpose the integrin $\alpha v\beta 3$ onto the viral capsid to obtain an approximate docking model for the FMDV–$\alpha v\beta 3$ complex. (B) Ribbon drawing of a FMDV protomer together with the docked $\alpha v\beta 3$ receptor (yellow). For clarity, only the β propeller of subunit α and the βA and hybrid domains of the subunid β are represented. The docking model suggests that the $\alpha v\beta 3$ receptor binds the FMDV G-H loop in an exposed position similar to that found when the loop is recognized by neutralizing antibodies. (See Color Insert.)

The genomic changes that can endow FMDV with the capacity to use alternative mechanisms of cell recognition (Section V,A) are minimal, and viruses with alterations in the RGD and with unusual receptor-binding specificities are likely to be present in the mutant spectrum of FMDV replicating in the animal host (Taboga *et al*., 1997) (described in Section IVB). The antigenic alterations produced by replacements at or around the RGD suggest that the emergence of these particular FMDV mutants *in vivo* was the result of selection of antigenic variants that escaped neutralization by anti-FMDV antibodies in peptide-vaccinated cattle (Fig. 4). A study analyzing the genetic changes selected during the adaptation of FMDV to guinea

pig documented the progressive dominance of an unusual amino acid replacement (Leu-147 to Pro) affecting the antigenic structure of the G-H loop of capsid protein VP1 in the course of adaptation of FMDV to this new host (Núñez *et al*., 2001). Construction of infectious cDNA clones of FMDV confirmed that this Leu-147 was essential for virus interaction with integrin receptor molecules expressed in BHK cells and various other cell lines used commonly to propagate FMDV and that mutants with Pro-147 do not form plaques on BHK-21 cells (Núñez *et al*., 2001). The isolation of FMDV mutants displaying altered cell tropism in association with antigenic changes illustrates the important adaptive potential of FMDV and the capacity of this virus to explore new antigenic/receptor recognition structures upon replication in its hosts.

A mutant of rabies virus harboring changes Lys-330 to Asn and Arg-333 to Met at antigenic site III of its surface glycoprotein manifested a modification in both cell tropism and antigenicity (Coulon *et al*., 1998). This double mutant, selected by the successive use of two neutralizing antiglycoprotein monoclonal antibodies, was not pathogenic for adult mice and could not penetrate the nervous system either by the motor or by the sensory routes. *In vitro* experiments showed that the double mutant was able to infect BHK cells, neuroblastoma cells, and freshly prepared embryonic motoneurons, albeit with a lower efficiency than the parental strain CVS. Upon further incubation at 37 °C, the motoneurons became resistant to infection by the mutant while remaining permissive to infection by the CVS strain (Coulon *et al*., 1998). Thus, rabies virus can use different types of receptors: a molecule that is expressed ubiquitously at the surface of continuous cell lines and that is recognized by both CVS and the double mutant and a neuron-specific molecule that is not recognized by the double mutant.

One of the first lines of evidence of coevolution of receptor usage and antigenicity in viruses was documented with human influenza type A with single amino acid replacements in the hemagglutinin, which modified receptor specificity or receptor-binding affinity (Robertson *et al*., 1987; reviewed in Skehel and Wiley, 2000). Additional cases of an overlap of receptor-binding and antigenic sites are given in Table IV.

B. Use of Soluble Receptor Analogs and Receptor Ligands, and Selection of Resistant Viruses

Ever since viral receptors were discovered, antiviral therapies have been designed based on the administration of soluble receptors and receptor ligands. The discovery that chemokine receptors act as cofactors essential for HIV entry into target cells identified new targets for

antiretroviral therapy. Viral entry can be inhibited *in vitro* by the natural ligands for CXCR4, the CXC chemokine SDF-1, and CCR5, the CC chemokines RANTES, MIP-1α and MIP-1β. Several peptidic compounds have been identified as CXCR4 antagonists that display anti-HIV activity, and the HIV-1 tat protein has been described as a "natural" CXCR4 antagonist with anti-HIV-1 activity. The bicyclam derivatives are among the most potent and specific CXCR4 antagonists described and they block X4 HIV replication very efficiently (De Clercq and Schols, 2001). Mutants resistant to CCR5 antagonists have been isolated. This resistance is not mediated by a change in coreceptor usage, but rather by the ability to use CCR5 despite the presence of the inhibitor (Trkola *et al.*, 2002). However, much remains to be learned about the possible physiological impact of the administration of receptor analogs and receptor ligands. The multitude of different receptors involved in HIV entry into cultured cells and their important physiological roles *in vivo*, together with their implications in distinct pathogenic processes, have added further complexity to this field of research (Heveker, 2001).

One of the most obvious potential difficulties with the use of receptor analogs as an antiviral therapy is the selection of soluble receptor-resistant mutants from the quasispecies swarms. This was illustrated with the avian sarcoma-leukosis virus subgroup A (ASLV-A), in which virus with a six amino acid deletion in the heterogeneous region 1 of the surface protein (SU) was selected by a soluble receptor in cell culture (Holmen and Federspiel, 2000). In some other instances, soluble receptors were capable of inducing conformational changes in viral envelopes, and nonsusceptible cells became susceptible to viral infection. In the case of ASLV-A, soluble Tva could activate SU and convert it to a fusogenic conformation-competent form capable of mediating the fusion of viral and cellular membranes, even when the membranes did not harbor viral receptors (Damico and Bates, 2000). This has also been observed with murine leukemia viruses (Lavillette *et al.*, 2000), with mouse hepatitis virus (Taguchi and Matsuyama, 2002), and with HIV-1 in that CD4 on the surface of a cell could be used by the virus to fuse to neighboring cells, provided the latter had a coreceptor (even in the absence of CD4) (Speck *et al.*, 1999).

There is no solid reason to assume that the selection of viruses resistant to receptor analogs or to receptor ligands should be substantially more difficult than the selection of antibody- or inhibitor-resistant mutants in the course of viral replication, selections that have been documented profusely not only for HIV but for many other RNA viruses (Table I) (reviewed in Domingo *et al.*, 2001). From the

mechanisms exploited by viruses to escape antibodies, CTLs, or inhibitors and from the molecular basis of fitness gain, a number of pathways for the selection of mutants resistant to soluble receptors (or analogs) are plausible. Viral mutants could be selected that display an increased affinity for the receptor attached to the cell surface, and not for its soluble form (or analog). A completely independent pathway could be the selection of mutants able to use an unrelated receptor, as this has already been described even for viruses not subjected to the presence of receptor analogs (Section V,A). Yet another possibility is the selection of mutants capable of binding both the cellular receptor and the authentic receptor on the cell, with a slight affinity bias to permit cell entry, among other conceivable variations of these mechanisms.

If an antiviral therapy based on soluble receptors, receptor analogs or receptor ligands is to be pursued, it may be necessary—in line with antiviral strategies based on combination therapy—to administer simultaneously several types of soluble analogs when potential alternative receptors for the virus have been identified. An obvious problem is the possible physiological impact of such a combination of receptors or their analogs. These difficulties should also be evaluated carefully if strategies employing combinations of inhibitors, receptor analogs, and immunotherapy are considered. Viruses have learned to confront many selective constraints over extended periods of coevolution with their hosts (Sections I and V) and it is not known whether artificial constraints may be found that would suppress viral replication in a way as to prevent selection of escape mutants.

C. Emergence and Reemergence of Viral Diseases

The emergence and reemergence of infectious diseases are increasing concerns for human and animal health and for a number of economically significant activities related to agriculture and farming. More than 40 emergent and reemergent human viral diseases have been described over the last two decades, and the adaptive potential of viruses, including changes in host range, undoubtedly has played a very relevant role (reviewed in Brault *et al*., 2002; Leitmeyer *et al*., 1999; specific recent examples in Morse, 1993; Morse, 1994). However, a complex set of interconnected influences are involved in viral disease emergence. These include environmental and climatic changes, socioeconomic and political factors, agricultural practices, and technological developments. The recent reemergence of the foot-and-mouth disease in Europe illustrates how a number of rather unpredictable and complex factors can produce a devastating animal

disease epizootic (Samuel and Knowles, 2001; Sobrino and Domingo, 2001). Additional examples have been documented and discussed (Domingo *et al.*, 2001; Mahy, 1997; Morse, 1993, 1994; Murphy and Nathanson, 1994). These multiple influences have, as a net result, an alteration of viral traffic and of the demography of viral vectors and susceptible hosts (Morse, 1994), with the many implications of alterations of viral ecology (Hurst, 2000).

A majority of the emergent and reemergent viral diseases are associated with RNA viruses, and frequently those displaying high recombination rates. Examples are the expansion of dengue fever and dengue hemorrhagic fever (Gubler, 1998), recent outbreaks of poliomyelitis (Kew *et al.*, 2002), and the appearance of severe acute respiratory syndrome (SARS). The emergence of HIV stands as the most significant introduction of a retrovirus into the human population, likely from several simian virus ancestors, and the virus is evolving continuously through mutation and recombination (Crandall, 1999). Poliovirus variation has been signaled as a major problem for the successful eradication of poliovirus (Nomoto and Arita, 2002). Furthermore, poliovirus and the C-cluster coxsackie A viruses are grouped together based on phylogenetic analyses. It has been suggested that if polioviruses were eradicated, some coxsackie A viruses may have an opportunity to modify their receptor specificity from ICAM-1 to CD155 (Table II) and evolve toward a polio-like virus (Nomoto and Arita, 2002).

Avian and equine hemagglutinins bind preferentially to *N*-acetyl sialic acid linked to galactose by α-2,3 linkages (Neu5-Acα-2,3Gal), whereas most human HAs bind preferentially to Neu5-Acα-2,6Gal. Changes in binding sites have been reported to cause host range switches among different hosts (Aytay and Schulze, 1991; Matrosovich *et al.*, 1997, 1999, 2000; Naeve *et al.*, 1984; Rogers *et al.*, 1983a, 1983b). The receptor-binding specificity of the avian influenza hemagglutinin was altered early after the transmission to humans and pigs (Matrosovich *et al.*, 2000), constituting a case of positive selection by the recipient host.

Modifications in host cell tropism need not be associated with a modification in receptor specificity. As discussed in Section V A, adaptation of a FMDV clone to the guinea pig resulted in the dominance of FMDV mutants with replacement Leu-147 to Pro in the G-H loop of VP1, which altered integrin recognition by the virus. However, the critical amino acid replacement for FMDV to cause disease in guinea pig was located in nonstructural protein 3A (Núñez *et al.*, 2001). It is not known what is the actual contribution of Pro-147 in VP1 to the

pathogenesis of FMDV in guinea pigs, but this model study with FMDV illustrates yet another possibility in a cascade of adaptive events: a virus may emerge as pathogenic in a new species without a strict requirement for a shift in receptor recognition, but in the course of replication in the new host, additional mutations that entail modifications in receptor recognition become permissible and perhaps favored (Núñez *et al*., 2001).

D. Gene Flow and Gene Therapy: Role of Viruses

The growing list of complete genomic nucleotide sequences of many cellular organisms and viral genomes suggests that all life forms share some basic functional motifs (Mount, 2001). No viral functions that depart in any outstanding way from similar cellular functions have been identified. This includes similarities between viral and cellular proteins involved in genome replication, proteolytic activities, and general patterns of genome organization and expression. These important advances in molecular genetics suggest that the exchange of modules, together with mutation, has contributed to the coadaptation of cells and autonomous replicons over long evolutionary periods (DeFilippis and Villarreal, 2001; Domingo, 2003; Domingo *et al*., 1999; Gorbalenya, 1995; Holland and Domingo, 1998). In cases in which viruses infect multiple host species, convergent phylogenies of viruses with their host can sometimes be seen (Gibbs and Weiller, 1999; McGeoch and Davison, 1999). Some of the features of variation of cell tropism discussed in Sections III,A and IV,A may not be foreign to differentiated organisms. The insertion of two amino acids into ectodysplasin—a member of the tumor necrosis-binding family—resulted in a change of its cellular receptor specificity, and the differential expression of the two forms of ectodysplasin plays a role in epidermal morphogenesis (Yan *et al*., 2000). Not only functional modules but also biological strategies are shared among viruses and cells.

In the case of viruses, the capacity to use alternative receptors and to change receptor specificities by modest genetic variation (involving short genetic distances) may represent an adaptation of viruses to cope with increasingly differentiated organisms. The absolute requirement of a cellular environment for virus replication (one of the definitive features of viruses; Section II,A) implies a permanent coexistence of cellular and viral genomes. The uptake of cellular genes by viruses has been documented in transducing bacterial viruses, RNA and DNA tumor viruses and, as remarkable cases, in cytopathic variants of the flavivirus bovine viral diarrhea virus, and defective viruses that have acquired host

sequences by nonhomologous recombination events (examples reviewed in Domingo *et al.*, 1999; Bushman, 2002; Domingo 2003).

The elucidation and interpretation of the complete genomic sequences of an increasing number of prokaryotic and eukaryotic organisms indicate that a substantial part of many of these genomes consist of mobile genetic elements or their relics (reviewed in McClure, 1999; Mount, 2001; Bushman, 2002). It has been estimated that this part of the human genome is 40% of the total! The process by which cells from one organism have captured DNA fragments from another organism is known as horizontal gene transfer. This transfer appears to be particularly frequent in prokaryotes, but it does also occur in eukaryotes. In addition to virus infection, lateral gene transfers are mediated by conjugative plasmids and several classes of mobile elements. Transfers are affected by viral infection (transduction), conjugation, and transformation. The transposition of sequences may occur within the DNA of the same cell.

Virus receptor specificity and its variations (as discussed in previous sections) may facilitate the access of cellular genes to specific cell types in tissues and organs. The human body comprises 50 trillion cells organized in hundreds of organs, tissues, and classes of cell subpopulations. Viruses may spread differentially within organs. As an example, the dissemination of neurotropic viruses in the brain is used increasingly to define connections among subsets of neurons (Card, 2001). To complete its incorporation into a new host cell, the transferred DNA must integrate into the recipient chromosome by some type of molecular recombination event. In this respect, integrative bacteriophages and retroviruses provide the adequate mechanisms as an integral part of their life cycles (Coffin *et al.*, 1997). The retroviral reverse transcriptases must jump strands to complete the synthesis of cDNA, and this property may favor the capture of cellular sequences for transfer to other cells. The mechanisms that mediate the integration of lysogenic bacteriophage DNA, retroviral cDNAs, and a class of transposons termed non-LTR retrotransposons are different, as are the proteins involved (reviewed in Ptashne, 1992; Bushman, 2002; Coffin *et al.*, 1997). Nonidentical requirements for integration may have broadened the possibilities of DNA flow among cells in the course of evolution. About 8% of the human genome appears to have been derived from retroviral-like sequences. Retroviral-mediated gene transfer can have deep phenotypic consequences for cells, such as a variety of disease manifestations in differentiated organisms (McClure, 1999), including alterations of cell growth control, and in some cases of infertility in humans (Sun *et al.*, 2000). Estimates using mice suggest that 40% of

DNA insertion events may have phenotypic consequences for the animal (reviewed in Bushman, 2002).

This brief account of the extensive evidence of lateral gene transfers in the cellular world underscores the profound evolutionary implications of the rapid changes in receptor specificities that viruses can undergo. Indeed, many of the viruses referred to in previous sections may infect multiple host species, and through genetic change they may acquire the potential to infect other host species, therefore permitting gene transfers among distant host species and, perhaps occasionally, also among taxa. Many lines of evidence (too numerous to be reviewed here) have clearly documented DNA transfers among distant domains of life (bacteria to plants, bacteria to animal cells, etc.) (reviewed in Bushman, 2002). It is not known how many DNA transfer events are initiated and never completed, and of those in which the transfer is completed, how many lead to viable genotypes. Perhaps the sorting of viable recombinant genomes is parallel on long evolutionary times to the sorting of viable RNA genomes subjected to mutational pressure that occurs over infinitely short evolutionary time scales (Section II,B). In both cases, negative selection may eliminate many nascent mutant and recombinant forms to leave a surviving minority endowed with a minimally required relative fitness value. Genetic disease in differentiated organisms may be viewed as an unavoidable price to pay to ensure a source of genome fluidity for long-term survival.

Gene therapy is a growing field of research in molecular medicine, whose aim is the supply of a functional gene into human cells for the treatment of inherited or acquired genetic disorders (Lemoine, 2000). The application of DNA recombinant techniques, together with an understanding of the life cycle of viruses, has permitted the development of virus vectors for the transport of foreign genes into target cells. This technology takes advantage of one of the natural tendencies of viruses to act occasionally as agents of horizontal gene transfers, although for therapeutic purposes, viruses must be modified carefully to act as safe and efficient vectors. Viruses that have been used as gene vectors include adenoviruses, parvoviruses (adeno-associated viruses), herpesviruses, retroviruses (simple and complex lentiviruses such as HIV), SV40, and some RNA viruses such as Sindbis virus (reviewed in Pfeifer and Verma, 2001). Viral vectors are engineered to have the foreign "transgene" replace deleted viral genes, while maintaining the *cis*-acting elements to form virions and to integrate the transgene. Packaging cells or plasmids provide *in trans* those proteins required for particle formation. A number of safety features (that necessitate

different approaches for the different vector types) must be introduced to avoid the spread of replication-competent viruses in the organism, cytotoxic effects, or undesired gene damage due to unrestricted and random integration of transgene into human DNA. Important difficulties encountered in viral-mediated gene therapy are the immune response (antibody or cellular responses evoked by the vector or preexistent in the human recipient), vector stability, dependence of the transduction efficiency on the metabolic state, and cell cycle phase of the target cells, among others. From the point of view of targeting the desired target cells, the receptor specificity of the viral vectors is an essential element of vector safety and efficacy, and many of the considerations discussed in previous sections concerning variations in receptor specificity are relevant to the design of viral vectors, both regarding the basal amplitude of host range afforded by each vector type and the possibilities of engineered alterations in the vector surface proteins to attain a more defined cellular specificity. Vectors based on adeno-associated virus type 2 or retroviral pseudotypes containing the surface glycoprotein of vesicular stomatitis virus display a broad host cell tropism. In contrast, vectors based on murine leukemia virus or HIV-1 often show restricted tropism, imposed by the receptors recognized by their surface envelope proteins (receptor types utilized by some viruses used for vector construction are listed in Table III). For some vectors, the host range can be expanded by replacing surface proteins (or subdomains) by heterologous counterparts, such as heterologous envelope proteins of retroviral vectors (Pfeifer and Verma, 2001).

VI. Conclusions and Overview

The picture beginning to form from genome analyses of viruses, unicellular organisms, and multicellular organisms is that viruses have shared functional modules with cells. A process of coevolution has probably involved exchanges of genetic information between cells and viruses for long evolutionary periods. From this point of view, it is perhaps not surprising that present-day viruses show flexibility in receptor usage and a capacity to alter through mutation their receptor recognition specificity. Shifts in receptor usage have been documented for a variety of DNA and RNA viruses, and the list is increasing continuously. This is just one facet of the fluidity exhibited by cells and viruses regarding exchanges and variation of their genetic material. This is particularly spectacular for RNA viruses because they cannot replicate without producing mutants. The mutation rates, mutation

frequencies, and population dynamics of DNA viruses during short-term evolution have been investigated far less than those of RNA viruses. For this reason, it is not possible to anticipate at present whether some DNA viruses may share with RNA viruses a rapid adaptation to using new receptors. It is possible that for the complex DNA viruses, due to a likely limited tolerance to generalized high mutation rates, modifications in receptor specificity will be less frequent than for RNA viruses, albeit with similar biological consequences once they occur.

RNA viruses consist essentially of pools of mutants, and minority components of such pools may rise to dominance under appropriate environmental conditions. Just by exploring constellations of modest numbers of amino acid substitutions in their capsid or envelopes, viruses can extend the range of receptors they can utilize or coutilize to enter the same cell type, a means for the virus to secure its survival. Different receptors, or allelic forms of one receptor, may be used with different efficiency, and receptor affinities are probably modified by mutation and selection. Receptor abundance and its affinity for a virus may modulate not only the efficiency of infection, but also the capacity of the virus to diffuse toward other sites of the organism.

The acquired ability of an RNA virus to enter a new cell type may have yet another consequence for the biological behavior of the virus progeny due to the new intracellular environment encountered by the virus in which the subsets of genomes undergoing positive or negative selection may be different than those that underwent the same processes in the previous host cell type. Therefore, a change in receptor specificity represents biological novelty not only in that a new cell type is infected, but also because the new environment may alter the mutant repertoire participating in subsequent rounds of infection. The application of microarray-based gene expression screening methods suggests considerable variation in expression patterns not only between different tissues and organs but also, on occasion, among cells of the same tissue. Thus, there is a great potential for environmental heterogeneity to modulate the composition of quasispecies swarms.

The main conclusion of this review is that receptors may be shared by different, unrelated viruses and that one virus may use several receptors and may expand its receptor specificity in ways that, at present, are largely unpredictable. We have suggested that this may have consequences for viral pathogenesis, coevolution of cell receptor specificity and antigenicity, a number of possible medical applications of receptor analogs and receptor ligands, the host range of viruses, the emergence of viral disease, and the application of viral vectors for viral therapy. Most of the studies on which we have based our arguments have been

carried out with a single virus isolate, clone, or reference strain. Findings may be dependent on a particular sequence context of the viral genome in view of the compactness of information in viral genomes and the connections among genomic regions. It would not be surprising if similar studies with other representatives of the same virus genus yielded different results in the detail of the specific mutations associated with a phenotypic change regarding receptor recognition. However, we would also expect that the results would still convey the same general concepts and conclusions, as they derive from the phenotypic flexibility resulting from high mutation rates and quasispecies dynamics.

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ADVANCES IN VIRUS RESEARCH, VOL 62

INFECTIOUS PANCREATIC NECROSIS VIRUS: BIOLOGY, PATHOGENESIS, AND DIAGNOSTIC METHODS

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I. Introduction

Infectious pancreatic necrosis virus (IPNV) is the etiological agent of an acute contagious systemic disease of several species of freshwater and marine fish, molluscs, and crustacean. The disease is widespread and the mortality in salmonids is inversely proportional to the age of the fish, being typically higher in fry and fingerling salmonids and relatively rare in older fish, in which infections can be frequently inapparent.

Interest in fish viruses has increased because aquaculture has expanded, intensified, and diversified worldwide during the past three

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decades and the movements of live aquatic animals and animal products have accelerated the accidental spread of diseases into new populations and geographical regions.

Global aquaculture production was estimated as 32.9 million tons in 1991, which represents 26.32% of the world production of fish, shellfish, and other aquatic organisms and has increased to reach 45 million tons in 1998 (FAO, 2000). The Asian region dominates the world in finfish culture (cyprinids mainly), producing about 90% of that produced globally, whereas North America or Europe contribute with only 2 and 5%, respectively, but the cultured fish species have a great commercial importance. Thus, Europe is the main producer of trout (54% of the global aquaculture production), turbot (100%), and sea bass/sea bream (68%) (Ariel and Olesen, 2002). Otherwise, the increasing consumer demand for salmonids and their products is expected to lead further growth in the salmonid industry. The world production of farmed Atlantic salmon is forecast to reach 1.3 million tons in 2005, with Norway producing 620,000 tons and Chile 300,000 tons.

These fish species are also very important from a sanitary perspective, as they are susceptible to microbial infections that may provoke disease outbreaks. Despite the strategies for sustainable expansion of aquaculture, disease outbreaks remain a considerable obstacle to aquaculture production and development. The economic impact of these diseases, especially those of viral etiology, has stimulated studies that provide a detailed account about the biology and pathogenesis of the viruses considered of serious concern.

One of them is IPNV, perhaps the most widespread of the piscine viruses. IPNV belongs to the genus *Aquabirnavirus* within the family Birnaviridae (van Regenmortel *et al*., 2000). The family contains three genera: genus *Aquabirnavirus* (type species, IPNV and yellowtail ascites virus) of fish, genus *Avibirnavirus* (type species, infectious bursal disease virus, IBDV) of birds, and genus *Entomobirnavirus* (type species, *Drosophila* X virus, DXV) of insects. Until now, human and mammals pathogens have not been recognized.

IPNV and IPN disease are unique in several aspects.

1. IPNV has a number of features not found in other double-stranded (ds)RNA-containing viruses: (i) the genome consists of two segments of dsRNA. Segment A is structurally and functionally bicistronic and contains a 2916 nucleotides (nt) large open reading frame (ORF) that encodes polyprotein pVP2-NS-VP3 (106 kDa) and a small ORF (444 nt) that encodes a 17-kDa arginine-rich minor polypeptide. The 106-kDa polyprotein is cleaved cotranslationally by the virus-coded protease

(NS) to generate the structural proteins VP2 and VP3 (Duncan *et al*., 1987). (ii) The presence of genome linked protein (VPg). Birnaviruses are the only RNA viruses with a viral RNA linked covalently to a polypeptide. VP1 is present in the virion in two forms, as a free polypeptide and as a VPg. (iii) The transcription occurs via a semiconservative mechanism, in contrast to the reovirus, in which transcription occurs by a conservative mechanism.

2. Birnaviruses are the most ubiquitous pathogenic microorganisms in aquatic species.

3. On addition to the high mortality of infected young salmonids IPN disease can have a significant economic impact; survivors of epizootic disease may become lifelong carriers, maintaining the virus in the population by continuously shedding and perpetuating the disease by vertical and horizontal transmission.

II. Biology of Infectious Pancreatic Necrosis (IPN) Virus

A. Virion Morphology and Biophysical Properties

Virions are unenveloped, single-shelled icosahedrons with characteristic isometric hexagonal profiles and have a diameter of about 60 nm (from 55 to 75 nm). The capsid consists of 180 structural subunits shared between 132 capsomers on the surface of the virion possessing a triangulation number (T) of 13 (Ozel and Gelderblom, 1985).

The virion possesses a sedimentation coefficient of 435 S and a buoyant density in CsCl and the empty capsid of 1.33 and 1.29 g/ml, respectively (Dobos *et al*., 1977). The molecular mass of the particle is 55×10^6 Da, and the estimated total mass of the capsid protein is 50.2×10^6 Da. The difference of 4.8×10^6 Da is due to the RNA component, which constitutes 8.7% of the particle weight (Wolf, 1988).

IPNV is acid, ether, chloroform, and glycerol stable and is relatively heat stable. IPNV is stable in storage at 4 °C for 4 months, but for long-term storage it should be held at −20 °C or lower. Thus far, it is the most stable of the fish viruses at salinities from 0 to 40%, and it is lyophilized readily in the presence of skim milk, lactose, or lactalbumin hydrolysate (Mortensen *et al*., 1998).

The infectivity of IPNV is unusually stable in filter-decontaminated water held at 4 °C and it persisted for at least 5–6 months (Baudonuy and Castric, 1977). In municipal tap water held at 10 °C, infectivity similarly persisted for more than 7 months, but at 10 °C in nontreated

natural water, virus was not detectable after only 14 days (Ahne, 1982). A comparable viral loss occurred in freshwater and seawater in only 17 days (Barja *et al.*, 1983; Toranzo and Hetrick, 1982). Infectivity persisted four times longer in sterilized estuarine water than in natural polluted water (Toranzo *et al.*, 1983).

IPNV is also very resistant to drying (4 weeks), γ irradiation (10% infectivity at 10^6 rads doses), and UV irradiation (5 min at 254 nm) (Ahne, 1982; Oye and Rimstad, 2001). The infectivity of IPNV is reduced by several disinfecting agents, such as chlorine, iodine, ozone, and formalin (Economon, 1973; Hamor, 1996; Liltved *et al.*, 1995). As might be expected, inactivation by pH 2.5 is incomplete, but pH 12.5 is virucidal (Wolf, 1988).

Most fish viruses are heat labile, but IPNV is an exception. In the range of pH 3.0 to 9.0, the rate of thermal inactivation at 60 °C is biphasic, with the most rapid activity occurring at pH 3.0 during the first 30 min. In the physiological range and at pH 9.0, significant infectivity was sustained for several hours (Desautels and MacKelvie, 1975).

B. Viral Genome

The genome, which represents 8.7% of the total virion mass, consists of two segments of double-stranded RNA (segment A = 2.5×10^6 Da, and segment B = 2.3×10^6 Da). The G-C content is 54% and the denaturation temperature is 89 °C.

Viral RNA is linked covalently to a 105-kDa polypeptide (VPg) (Persson and MacDonald, 1982) that it is identical to the VP1 polypeptide (94 kDa). Therefore, the VP1 polypeptide is present in the virion both in a free and in a genome-linked form. The 5′ end of each RNA strand is linked to a serine residue in the VPg by a phosphodiester bond (Calvert *et al.*, 1991).

Genome segment A (between 2962 and 3104 nt) contains a large ORF encoding a 106-kDa polyprotein (PP) (NH_2-preVP2-NS protease-VP3-COOH), which is cleaved cotranslationally to generate three polypeptides: pVP2, the precursor of the major capsid protein VP2; VP3, a minor capsid protein; and NS, a nonstructural protein. The pVP2 protein is further cleaved to VP2 during virus maturation. The protease activity responsible for the cleavage has been associated with the nonstructural virion protein NS (Duncan *et al.*, 1987; Magyar and Dobos, 1994a; Manning and Leong, 1990; Manning *et al.*, 1990). Genome segment A contains an additional small ORF, which precedes and partially overlaps the amino-terminal end of the polyprotein ORF

and it is in a different reading frame (Duncan *et al*., 1987). This small ORF encodes a 17-kDa arginine-rich minor polypeptide (VP5) with unknown function, which has been detected in infected cells but not in purified virions (Magyar and Dobos, 1994b). Suzuki *et al*. (1998), sequencing the VP5 gene of a marine birnavirus, suggested that VP5 is a basic protein that may function in RNA binding.

The product of genome segment B (between 2731 and 2784 nt) is a minor internal polypeptide VP1. This protein constitutes the putative virion dsRNA-dependent RNA polymerase (RdRp) (Duncan *et al*., 1991). VP1 is present in the virions in the two forms mentioned earlier: as a free polypeptide and as a genome-linked protein.

C. Protein Composition

The main structural proteins of IPNV, analyzed by polyacrylamide gel electrophoresis (PAGE), are distributed into three size classes, which occur in different relative proportions: one large virion polypeptide VP1 (94 kDa, 4% mass of virion protein), one medium-size polypeptide VP2 (54 kDa, 62%), and two small polypeptides VP3 (31 kDa, 28%) and VP3a (29 kDa, 6%) (Dobos, 1995a).

VP1 is the RNA polymerase and is derived from monocistronic translation of the small RNA B segment. VP2 and VP3 proteins are derived from proteolytic cleavage of a polyprotein precursor encoded by a large RNA A segment (Duncan *et al*., 1987).

VP2 is the outer capsid protein and contains neutralization epitopes (Lipipun *et al*., 1992) and cell attachment sites that determine host/cell range. It has been demonstrated that two variable and one conserved neutralization epitopes are localized within VP2 (Caswell-Reno *et al*., 1986; Christie, 1990; Frost *et al*., 1995).

VP3 and VP3a have been described with a complete or essentially internal localization. Dobos (1977) detected only VP2 but not VP3 from a purified empty capsid labeled isotipically using PAGE and autoradiography. On the basis of these results, VP3 was proposed to be an internal polypeptide (Park and Jeong, 1996), associated with genomic material forming the unique thread-like rinonucleoprotein complexes (Hjalmarsson *et al*., 1999). However, Nicholson (1993) suggested that at least a portion of VP3 may be exposed on the virion surface, as using both ELISA and immunodot assays the virus was reacted with a number of anti-VP2 and anti-VP3 monoclonal antibodies (mAbs).

The major polypeptides are easily detectable by PAGE and stain with Coomassie blue, but the minor proteins of IPNV required other,

more sensitive detection methods. These minor polypeptides and their detection methods are the following.

i. A 49-kDa polypeptide is detected by silver staining, by autoradiography, or by the use of immunological methods. The 49-kDa polypeptide has shown to represent an amino-truncated VP2 (Duncan *et al*., 1987).
ii. The PP is detected by Western blotting using anti-VP2 and anti-VP3 mAbs (Magyar and Dobos, 1994a).
iii. The NS polypeptide is a nonstructural protein that reacts with polyclonal anti-IPNV serum in Western blots (Manning and Leong, 1990). Havarstein *et al*. (1990) detected a truncated NS polypeptide that designated VP4, which represents the virion-associated truncated NS polypeptide.
iv. The presence of the 17-kDa polypeptide in purified virus is still uncertain. Havarstein *et al*. (1990) detected a band in the 17-kDa regions in autoradiograms of purified labeled IPNV that they named VP5.

Several authors have investigated the glycosylation of viral polypeptides. The IPNV segment A sequence indicates four potential *N*-glycosylation sites in the pVP2 gene in the serotype Jasper (Dobos, 1995a) and six in IPNV Sp and N1 (Havarstein *et al*., 1990). No evidence of VP2 glycosylation in IPNV has been obtained previously (Heine *et al*., 1994; Perez *et al*., 1996), although Hjalmarsson *et al*. (1999) demonstrated that VP2 specifically binds lectins recognizing sugar moieties of *N*-acetylgalactosamine, mannose, and fucose. The metabolic inhibitors of *N*-linked glycosylation did not prevent the addition of sugar residues to virions. These results suggest that the glycosylation of VP2 is of the *O*-linked type when IPNV is propagated in RTG-2 cells.

Regarding other polypeptides, there are two potential glycosylation sites near the amino termini of VP3 of IPNV N1, and eight and six potential glycosylation sites of the VP1 coding region of IPNV Jasper and Sp, respectively (Duncan *et al*., 1991). However, there have been no suggestions that VP3 and VP1 are glycosylated.

D. Virus Replication

Most of the knowledge on the molecular biology of IPNV is due to the continuous work of Dobos and co-workers, who have being studying the features of the genome and viral replication process since the early 1970s. Their work is compiled in an excellent review (Dobos, 1995a).

IPNV and aquatic birnaviruses are replicated routinely in a variety of established fish cell lines, such as BF-2 (bluegill fry), CHSE-214 (chinook salmon embryo), EPC (epithelioma papulosum cyprini), RTG-2 (rainbow trout gonad), and SAF-1 (sea bream fins) (Ahne, 1977; Bejar *et al*., 1997; Wolf, 1988). The optimum temperature for IPNV growth is 20 to 24 °C (Dobos *et al*., 1977). Lorenzen *et al*. (1999) demonstrated that BF-2 and CHSE-214 cells yielded the best results for the isolation and *in vitro* culture of IPNV.

The virus replicates in the cytoplasm, and a single cycle of replication takes 16–20 h at 22 °C, resulting in a characteristic cytopathic effect (CPE) in susceptible cell cultures (Malsberger and Cerini 1965) and in infectivity yields of 10^6 to 10^9 PFU/ml. However, cell lines can also be infected persistently and not show CPE. MacDonald and Kennedy (1979) and Hedrick and Fryer (1981) demonstrated that defective interfering (DI) particles usually appear in cell cultures infected at higher virus titers and are responsible for the development of persistently infected cells.

1. Attachment, Penetration, and Uncoating

The purified virus binds to high molecular mass polypeptides (100–200 kDa) of the cellular membrane of fish cells, both specific and nonspecific, and they constitute the virus receptors (Kuznar *et al*., 1995). Binding experiments have shown that the virus saturates the binding sites after 2–3 h at 4° C and that VP2 is the cell attachment protein of the virion (Dobos, 1995a).

There is little information on penetration or uncoating, but because Cohen (1975) found that the virion-associated RdRp is active without any proteolytic pretreatment of the virus, uncoating may not be a precondition of virus replication. More recent studies have shown that shortly after adsorption, the virus is internalized into vesicular peripheral compartments of the cell, suggesting that the entry of IPNV, as that of many other naked viruses, is attained by endocytosis (Couve *et al*., 1992). On the basis of these results and the fact that IPNV multiplication was sensitive to agents that neutralize the endosomic pH (Farias *et al*., 1988), Kuznar *et al*. (1995) hypothesized that endosomic pH promotes the changes required to let the virions escape toward the cytosol. Later, these authors demonstrated that the acid pH of endosomes seems not to be a mandatory condition to gain entrance into the cytosol of infected cells (Espinoza and Kuznar, 1997). Carrasco (1994) proposed that viruses require an energetic supply to translocate virions or genome complexes from endosomes (or the surface of the cell) to the cytosol. How pH, for instance, can have a dual role: it induces

changes in the structure of the virion and provides the pH gradient necessary as an energetic source for virion translocation.

2. RNA Synthesis

Somogyi and Dobos (1980) determined that the rate of IPNV-specific RNA synthesis in infected fish cells was maximal at 8–10 h after infection and decreased after 14 h. These authors detected three forms of RNA intermediates: (i) a putative 14–16 S transcription intermediate (TRI), which after RNase treatment comigrated in polyacrylamide gels with virion dsRNA; (ii) two species of 24 S RNase-sensitive, polysome-associated RNA (the viral mRNA) that could be hybridized to the two genome segments; and (iii) a 14 S dsRNA component undistinguishable from virion dsRNA by gradient centrifugation and PAGE. *In vitro*, unlike reovirus (Schonberg *et al.*, 1971) IPNV-infected cell transcription by the virion RdRp is primed by VP1 and proceeds via an asymmetric, semiconservative, strand-displacement mechanism (Dobos, 1995b).

Magyar *et al.* (1998) reported the presence of VP1–oligoribonucleotide complexes from infected cell lysates in which the RNA moiety represented 5′-terminal sequences of plus strands. Part of these complexes could be chased via replicative intermediates and genome-length single-stranded (ss)RNA to intracellular VP1-dsRNA and finally to virion VPg-dsRNA. These results are compatible with the theory that, as *in vitro*, a VP1–pG complex may act as a primer for viral plus RNA synthesis *in vivo*. However, it is important to underline the uniqueness of birnaviruses because they are the only dsRNA viruses with a genome-linked protein that exhibit protein-primed RNA synthesis (Dobos, 1993).

3. Protein Synthesis

Dobos and co-workers have extensively studied the time course of the synthesis of virus-specific proteins in fish cell cultures infected by IPNV. The results obtained showed that the four primary gene products designated as infected cell proteins (ICPs), such as ICP 94 kDa (the intracellular equivalent of VP1), ICP 62 kDa or pVP2, ICP 31 kDa (the intracellular equivalent of VP3), and ICP 29 kDa (the NS virus-coded protease), were produced in the same relative proportions throughout the infectious cycle between 3 and 14 h postinfection (pi) (Dobos, 1977). The rate of ICP synthesis increased during the first 6 h pi, reached its peak between 6 and 9 h pi, and declined thereafter.

Then, ICP 62 is cleaved posttranslationally via a 60-kDa intermediate to generate the major capsid polypeptide VP2. Part of ICP 31 is cleaved during maturation to generate a 29-kDa polypeptide, and both

the precursor (ICP 31) and the product (29 kDa) are found in the virion as VP3 and VP3a. ICP 29 (the NS protease) is degraded slowly into smaller polypeptides via a 25-kDa intermediate (Magyar and Dobos, 1994b).

The genome segment B encodes VP1 and segment A the other three primary gene products. However, the exact molecular mechanism is still under controversy. Somogyi and Dobos (1980) suggested the transcription of subgenomic mRNAs from genome segment A, and Dobos (1977) proposed a polyprotein processing or an internal initiation of translation on the segment A-specific mRNA. Mertens and Dobos (1982) suggested the presence of a polycistronic mRNA. Finally, Manning and Leong (1990) and Dobos (1995a) considered two models to understand the synthesis of these proteins: (1) independent translational initiation for each viral protein and/or (2) rapid proteolytic cleavage by a viral or host protease.

Nucleotide and peptide sequence analyses have shown that the large ORF of the A segment of IPNV is monocistronic and encodes a polyprotein in which three viral polypeptides are arranged in the order N-pre VP2-NS-VP3-C′ (Dobos, 1995a; Duncan and Dobos, 1986; Nagy *et al*., 1987). The product of genome segment B is a RNA polymerase (VP1) of IPNV. This polypeptide is present in the virion both as a free polypeptide and as a genome-linked protein (VPg).

4. Viral Assembly and Release

The assembly of virion components takes place in the cytoplasm of infected cells and virus release occurs via cell destruction. Hjalmarsson and Everitt (1999) identified the IPNV components released from productively infected RTG-2 cells following a massive cytopathic effect. The analysis of virus pools showed a mixture of polypeptides VP2 and VP3 but not the presence of preVP2; therefore, the subviral particles are not assembly forms of the precursor preVP2 of the major capsid protein.

Wu *et al*. (1998), studying protein maturation in IPNV-infected CHSE-214 cells, demonstrated that maturation of the capsid protein (VP2) and cleavage of VP4 (NS proteinase) could be blocked by serine proteinase inhibitors. The temporal and subcellular localization of IPNV structural proteins has been studied by Espinoza *et al*. (2000).

E. Serological Characterization

The serological relationships among aquatic birnaviruses are complicated, and the problems of serological classification are due to the lack of standardization and uniformity in the analytical methods.

Serological characterization of this group of viruses is very important for epizootical reasons as well as for the development of vaccines. There are excellent reviews on the serological characterization of birnaviruses (Hill and Way, 1983, 1995; Reno, 1999; Sadasiv, 1995) consequently, this review carries out a short summary on this topic.

IPNV comprises an antigenically diverse group of viruses. The first scheme for grouping the isolates proposed three major serotypes designated as VR-299 (serotype 1), Ab (serotype 2), and Sp (serotype 3) (Hill, 1977; Nicholson and Pochebit, 1981). Later, using cross-neutralization assays with almost 200 isolates of IPNV and other aquatic birnaviruses, Hill and Way (1995) divided the isolates into two distinct serogroups, which do not cross-react. Serogroup A contains nine different serotypes and most of the isolates associated with fish diseases, and the proposed nomenclature for the recognized serotypes is as follows: A1 (archetype West Buxton or WB), A2 (Sp), A3 (Ab), A4 (He), A5 (Tellina or Te), A6 (Can. 1), A7 (Can. 2), A8 (Can. 3), and A9 (Jasper or JA). Serogroup B consists of a single serotype (B1) with a smaller number of isolates, including the former serotype TV-1. The serotype N1 is not included in this serological classification because it was demonstrated that N1 is related to the Sp serotype (Melby and Christie, 1994). This new simplified terminology has been widely accepted.

As described earlier, serotyping of aquatic birnaviruses has been achieved by serum neutralization using polyclonal rabbit antisera against recognized reference serotypes. However, conflicting results have been caused by the lack of standardization of the methods used and by the differences in the specificity of the antibodies produced. mAbs offer a potentially attractive alternative, as they are of uniform quality in terms of specifity for particular epitopes of antigens and can be produced in large and standardized quantities. mAbs for IPNV and other aquatic birnaviruses were the first developed for fish viruses (Caswell-Reno *et al*., 1986, 1989), and a review of their production and current applications has been reported by Nicholson (1993). Some of the mAbs produced react with epitopes on VP2 (the major capsid protein) and usually possess neutralizing activity, whereas other mAbs react only with epitopes on the smaller internal structural protein VP3 and usually show no neutralizing activity.

Several studies on the antigenic relationships between the reference virus strains have been carried using mAbs capable of neutralizing IPNV infectivity (Caswell-Reno *et al*., 1986; Shankar and Yamamoto, 1994). These studies have resulted in the forming of subdivisions within the serotypes (subtypes) through the use of panels of mAbs,

each of which shows specificity for a single epitope on the VP2 or the VP3 protein.

The degree of antigenic variation observed among birnavirus strains is dependent on the number of the different mAbs used and the number of reference strains utilized to produce the mAbs (Hill and Way, 1995). Thus, Caswell-Reno *et al*. (1989), using a total of 11 mAbs in three panels, could distinguish 7 of the 9 serotypes previously established, but failed to distinguish between A7 (Can. 2) and A8 (Can. 3) serotypes. Using a total of 22 mAbs in three panels, Tarrab *et al*. (1993) could also differentiate 7 of the 9 serotypes but failed to distinguish between the A3 (Ab) and the A5 (Te) serotypes. More recently, Frost *et al*. (1995), using five mAbs, could only distinguish 5 of the 9 serotypes; they failed to differentiate between A3 (Ab) and A6 (Can. 1) and also between the A7 (Can. 2) and the A8 (Can. 3) serotypes.

For general serotyping purposes, Hill and Way (1995) suggested the use of a panel of selected mAbs, which would differentiate all nine serotypes, for example, combining the panels used by Caswell-Reno *et al*. (1989) and by Tarrab *et al*. (1993). In addition, the inclusion in the panel of a broadly reacting mAb such as the N1-C12 mAb developed by Christie *et al*. (1990) would recognize all virus isolates belonging to serogroup A. The first licensed mAb kits for these viruses have been those of Nicholson and co-workers (Nicholson, 1993).

Genetic relationships of aquatic birnaviruses have been studied at the genomic level. Some discrepancies exist between results of the serological assay and those of nucleotide sequencing. Comparing deduced amino acid sequences of a 310-bp cDNA fragment located at the junction of the C terminus of the pVP2 and the NS coding regions of the 17 isolates, Heppell *et al*. (1993) found only three major genogroups among the viral strains of serogroup A. No correlation was found among genogroups based on these VP2/NS cDNA sequences and the established serological groups. Furthermore, Sp and Ab were classified in different serotypes in the serological scheme of Hill and Way (1988), whereas studies of the nucleotide sequence (VP2/NS sequence) revealed that both strains should belong to genogroup II (Heppell *et al*., 1993).

Blake *et al*. (2001) compared the deduced amino acid sequences of a 2904 genomic fragment of genome segment A of nine aquatic birnaviruses strains of serogroup A. These strains were clustered into six genogroups. In contrast to previous studies based on shorter genomic sequences within the pVP2/NSs coding region (310 bp), genogroups based on the entire VP2 coding region correlated with serological classification.

F. Host Range

From its first isolation in the United States in 1957, IPNV has been detected in a number of different salmonid species, and throughout the 1960s it seemed that infections were limited to such salmonid fish (Wolf and Quimby, 1962, 1971). However, Sonstegard *et al.* (1972) reported the isolation of a birnavirus IPNV-like from healthy white suckers (*Catostomus commersoni*) in Canada, and Sano *et al.* (1981) detected a birnavirus from Japanese eels (*Anquilla japonica*). From these first evidences of birnavirus-induced disease in nonsalmonid fish species, birnaviruses and IPNV have been isolated from many different and increasingly diverse species of fish from freshwater, brackish, and marine environments, as well as from marine molluscs and crustacean.

The host range of aquatic birnaviruses has been reviewed by Hill (1982) and later by Ahne (1985), Wolf (1988), and Reno (1999). The known host range has expanded even more and currently comprises fish from more than 30 families and from more than 10 species of molluscs and several species of crustacean. In addition, Lo and Wang (1984) isolated a birnavirus from a trematode parasite of fish.

New fish hosts, including several species of aquacultural importance, have been described more recently, such as Senegalese sole (*Solea senegalensis*) (Rodriguez *et al.*, 1997); sea bream (*Pagrus aurata*) (Rodger *et al.*, 1997); rockfish (*Sebastes schlegeli*) (Joh *et al.*, 1999); ayu (*Plecoglossus altivelis*) (Jung *et al.*, 1999); Japanese eels (*Anquilla japonica*) (Lee *et al.*, 1999); and wild flounder (*Rhombosolea tapirina*), spikes dogfish (*Squalus megalops*), and ling (*Genypterus blacodes*) (Crane *et al.*, 2000).

There has been some confusion and inconsistency in the literature about the use of the term "IPNV" because some authors use it in a generic sense for any isolate related serologically to reference strains of IPNV irrespective of the host. According to Hill and Way (1995) and Reno (1999), the term IPNV must be applied strictly to describe those isolates shown to produce disease in salmonid fish, using the term "aquatic birnaviruses" for those that are avirulent for rainbow trout and other salmonid fish, and are usually isolated from other teleosts, or from aquatic invertebrates. All aquatic birnaviruses are similar in morphology and biochemical and biophysical properties (Dobos *et al.*, 1979).

III. Infectious Pancreatic Necrosis Disease

A. Disease Features

Aquatic birnaviruses are the most pervasive pathogens of aquatic animals. They have been isolated from teleosts as well as aquatic invertebrates of freshwater brackish and seawater environments.

1. Clinical Signs and Gross Pathology

The disease infectious pancreatic necrosis was regarded as a serious disease affecting mainly young rainbow trout held under intensive rearing until 1985, where severe mortalities due to IPNV were observed in Atlantic salmon postsmolts (Ariel and Olesen, 2002). The typical symptoms of the disease in salmonids possess the features of acute catarrhal enteritis (Wood *et al*., 1955). However, different strains of aquatic birnaviruses in other freshwater and marine fish species may cause different diseases, such as nephroblastoma and branchionephritis in Japanese eels (Egusa, 1970; Sano *et al*., 1981), gill disease in Japanese eels (Lee *et al*., 1999), ascites in yellowtail *Seriola quinqueradiata* fingerlings (Sorimachi and Hara, 1985), spinning disease of Atlantic menhaden (*Brevoortia tyrranus*) (Stephens *et al*., 1980), and renal necrosis in turbots (*Scophthalmus maximus*) (Castric *et al*., 1987). Reno (1999) summarized all the families of salmonid and nonsalmonid fish in which one or more species had been found to be infected or a carrier of IPNV.

IPN disease is characterized by behavioral changes and by gross internal and histopathological lesions, although there are no specific pathognomical signs of IPN disease. Behavioral changes include anorexia and an agonal corkscrew swimming motion interspersed with ataxia (Wood *et al*., 1955). The affected fish exhibit a variety of external signs of disease, such as a darkened pigmentation of skin, abdominal swelling, mild or moderate exophthalmia, paleness in gills, and sometimes hemorrhages in ventral areas and fins (Wolf, 1988).

Internally, lumens of the stomach and intestine are devoid of food but characteristically contain a clearly pathognomic milky cohesive mucus. In some infected fish, the pyloric caecal and anterior adipose tissue are flecked with petechiae, and the body cavity contains ascitic fluid. Spleen, heart, liver, and kidneys are often enlarged and abnormally pale, and petechial hemorrhages are observed throughout the visceral mass.

Mortality due to IPN is higher in young fish at ages below 6 months and rare in older fish in which infection are often inapparent (Frantsi and Savan, 1971). In salmon farming, IPN causes mortality in fry at start feeding, juveniles during smolting, and in postsmolts during the first months after sea transfer (Jarp *et al.*, 1994; Melby *et al.*, 1994; Smail *et al.*, 1992). The cumulative mortality of postsmolts during the first 3 months after sea transfer was found to vary from a low percentage to 80% of fish (Krogsrud *et al.*, 1989; Jarp *et al.*, 1994).

Several authors have attempted to determine if viral isolates from nonsalmonids species are capable of inducing IPN disease in salmonids and have standardized techniques and challenge protocols (Bootland *et al.*, 1986; McAllister and Owens, 1986). In some cases, aquatic birnaviruses from nonsalmonids could produce a typical IPN disease and mortality in salmonids (Hill, 1982; McAllister and McAllister, 1988; Perez-Prieto *et al.*, 2001a; Silim *et al.*, 1982). Nevertheless, birnaviruses from eels were unable to induce disease in trout, although they were virulent in homologous species (Sano *et al.*, 1981).

2. Histopathology

IPN disease produces several histopathological changes in the affected salmonids. Thus, severe and massive necrosis in acinar cells with nuclear pyknosis, karyorrhexis, and occasional basophilic cytoplasmic inclusions are observed in pancreas (Smail *et al.*, 1995; Taksdal *et al.*, 1997). The renal excretory and hematopoietic tissues are affected of congestion and hemorrhagic zones in glomeruli, edema, and destruction of tubule epithelium (Sano, 1971, 1973).

Mucosa of the pylorus, pyloric caecea, and anterior intestine show acute enteritis in the form of necrosis and sloughing of the epithelium (McKnight and Roberts, 1976; Smail *et al.*, 1995). These symptoms are characteristics of acute enteritis and might be a more lethal change than the necrosis of the pancreas (Wolf, 1988).

Rainbow trout and Atlantic salmon affected by IPN may also show congestion and necrosis of liver tissues (Sano *et al.*, 1971; Taksdal *et al.*, 1997), with focal degeneration of liver parenchymal cells (Swanson and Gillespie, 1979). The virions may be observed inside hepatocytes (Kudo *et al.*, 1973).

At the ultrastructural level, IPNV is observed both in pancreatic and kidney tissues (Lightner and Post, 1969; Yamamoto, 1974). Viral aggregates in the crystalline array covered by a membrane are detected inside the cytoplasm of infected cells. Viral titers in the tissues of infected fish are usually high in the range of 10^7–10^{10} tissue culture infective dose ($TCID_{50}$) per gram (Castric *et al.*, 1987).

B. Epizootiology of IPN

1. Factors Involved in the Disease

In hatchery trout and salmon, infection varies from inapparent or subclinical, in which losses are not important, to acute outbreaks, in which mortality affects nearly the total fish population. The severity and cumulative mortalities of IPN infection in salmonids depend on the combination of a number of factors related to the host, the virus, and the environment (Dorson and Torchy, 1981).

a. Host Factors Specific differences in disease resistance have been demonstrated among several fish species and among salmonids. Brook and rainbow trout have been found to be the most sensitive to IPN (Pilcher and Fryer, 1980), and the Atlantic salmon is the most resistant of the salmonid species studied to the lethal effects of the virus (Sadasiv, 1995). In addition, variations in IPN resistance have been found among cultured varieties of rainbow trout (Hill, 1982; Okamoto *et al*., 1987, 1993). Ozaki *et al*. (2001) identified two chromosomal regions containing quantitative trait loci (QTLs) in rainbow trout that were associated with IPN disease resistance/susceptibility.

An important influence of the host age on the virulence of infection for IPNV has been recognized by several authors, who established that the lethality of IPNV is higher in younger fish (Wolf *et al*., 1960b). In experimental infections of brook trouts, Frantsi and Savan (1971) obtained cumulative mortalities of 85% in 1-month-old fish, 73% in 2-month-old fish, 45% in 4-month-old fish, and negligible mortalities in 6-month-old fish within a 60-day observation period. In a similar study with rainbow trout, Dorson and Torchy (1981) obtained cumulative mortalities of 70% in 1- to 2-week-old infected fish but a very low percentage in 20-week-old infected fish. Older fish (over 5–6 months) are resistant to the disease and can become asymptomatic carriers of IPNV, shedding the virus periodically in feces and reproductive products (Ghittino *et al*., 1984; Wolf *et al*., 1963).

b. Virus Factors IPNV isolates show a wide variation in the pathogenicity degree. Wolf *et al*. (1963) demonstrated differences in virulence with 15 isolates of serotype A1 infecting brook trout fry, with mortalities ranging from 7 to 98%.

High virulent isolates cause a higher than 90% mortality in rainbow trout fry, but avirulent isolates cause no mortality (Sano *et al*., 1992). The serotype Sp is usually the most virulent, whereas the serotype Ab displays a low virulence degree (Jørgensen and Kehlet, 1971).

However, virulent and avirulent isolates are present within the same serological group (Hedrick and Okamoto, 1983; Sano *et al.*, 1990).

Some of the viruses from nonsalmonid fish and shellfish have been found to be pathogenic for rainbow trout fry in which they produce typical IPN disease (Hill, 1982, 1995). Pathogenicity for the host and cell tropism are determined by the interaction of viral proteins encoded by RNA segment A of IPNV (Sano *et al.*, 1992).

c. Environmental Factors Intensive culture systems are a highly stressful environment for fish. Stress in fish can cause immunosuppression and results in an increased susceptibility to disease or leads to an activation of persistent infections. Clinical IPN in hatchery-reared fry occurs mainly when fish are under stress due to adverse environmental conditions provoked by several mechanisms, such as rapid temperature changes, handling, crowding, high population density, and deterioration of water quality (low oxygen content, high ammonia, etc.) (Jarp *et al.*, 1994; Snieszko, 1974; Wedemeyer, 1970).

Water temperature is an important factor in the development of most fish diseases. Thus, the physiological responses of fish, including the defense mechanisms, are dependent on the fish body temperature that is always close to that of their environment (range 8–12 °C). Frantsi and Savan (1971) obtained cumulative mortalities of 74% at 10 °C, 46% at 15.5 °C, and no mortalities at 4.5 °C in brook trout fry infected with IPNV. Dorson and Torchy (1981) observed that the mortality rate in rainbow trout fry held at 6 °C was much lower than at 10 °C and was almost totally suppressed at 16 °C.

2. Pathogenesis

Experimental infection trials of IPNV using fry trout indicate that there is a latent period of about 2 days postinoculation in which IPNV into the water can be detected. Afterward, there is a period of 2 to 4 days postinoculation in which the clinical signs of the disease become apparent (Swanson and Gillespie, 1982). However, the incubation time of IPN depends on several factors, such as host age, water temperature, and fish species.

The pathogenesis of the IPN disease is not well established yet. Swanson (1981), studying the course of IPNV infection in young brook trout exposed to virus by intraperitoneal injection and oral inoculation, demonstrated that IPNV enters the peritoneal cavity of fish and interacts with pancreatic exocrine cells within 2 days. However, whether the virus enters the pancreas directly through the serosa or is carried there via the blood has not been determined yet. The pancreas seems to

be the main target for virus replication, although viral antigen and slight pathological changes were observed in the kidney and liver of some infected fish (Swanson, 1981; Swanson *et al*., 1982).

3. Transmission

The transmission of IPNV among fish hosts needs to be understood in order to suppress the disease provoked by the virus and to reduce the spread of the virus in aquaculture environments. There is accumulating evidence that the mode of transmission within a hatchery may be a combination of vertical and horizontal transmission.

a. Horizontal Transmission Horizontal transmission can occur by direct contact among healthy and infected fish, by ingestion, and by fomites.

Fish-to-fish transmission through the water is the major mechanism of spread between fry. During IPN epizootics, IPNV concentrations in water may reach levels as high as 10^5 $TCID_{50}$/ml (Desautels and MacKelvie, 1975), which are more than sufficient to infect fish and fish eggs (Ahne *et al*., 1989). Waterborne virus may also be accumulated in filter-feeding animals, and several studies show that marine bivalve molluscs may act as reservoirs of IPNV in the natural environment (Dobos *et al*., 1979; Lo *et al*., 1988; Mortensen *et al*., 1990, 1998).

Once IPNV has been introduced into a fish population, horizontal transmission occurs as a result of ingestion of contaminated feces, urine, water, or other materials and by contact of contaminated water with the gills of healthy fish (Frantsi and Savan, 1971).

b. Vertical Transmission The transmission of virus during spawning, regardless of whether the virus is located within or external to eggs, was suspected early in the history of IPN disease (Snieszko *et al*., 1957). Wolf *et al*. (1963) demonstrated the presence of high levels of IPNV in ovarian fluid and observed that some virus remained associated with washed ova. IPNV has been isolated repeatedly from sex products by several authors (Ahne, 1983; Ahne and Negele, 1975a, 1975b; Fijan and Giorgetti, 1978; McAllister *et al*., 1987; Mulcahy and Pascho, 1984, Rodriguez *et al*., 1992).

The transmission of IPNV inside eyed eggs was deduced when transmission to fry was not eliminated by the external disinfecting of eggs from infected brook stock (Bullock *et al*., 1976). Ahne and Negele (1985) isolated IPNV from eggshells after fry hatched and suggested that the virus might be protected in lobes and pores of the eggshells and adheres strongly to the chorion of water-hardened eggs. The

chorion of unfertilized eggs is smooth, but hardened eggs have a rough, lobe-like and porous texture, which would provide anchorage for the virus and may prevent disinfectants from reaching the virus.

True vertical transmission through virus contained within oocytes and sperm has not been demonstrated (McAllister, 1993). However, young salmonid fish may become infected by eating the eggshells at the time of first feeding or the virus may be carried on sperm (Sadasiv, 1995). Furthermore, the virus can adsorb to the spermatozoa (Mulcahy and Pascho, 1984) and penetrate the egg at fertilization. Dorson and Torchy (1985) obtained a successful transmission when spermatozoa were incubated with a relatively high concentration of IPNV. The transmission obtained via infected sperm was not prevented by disinfections after hardening (50 ppm iodine), which clearly demonstrated that IPNV was carried inside the egg by the spermatozoa (Bullock *et al*., 1976).

c. Carrier State Surviving fish of IPN epizootics develop a persistent infection or carrier state, which serves to continually transmit the infection to other fish (Bootland *et al*., 1991; Dorson, 1982; Wolf, 1988).

In natural salmonid populations, intermittent shedding by carriers is thought to be an important mechanism by which the virus ensures endemic persistence (McAllister, 1993). Several factors, including age, species of fish, water temperature, and strain of virus, can influence the severity of infection and the subsequent establishment of the carrier state. For instance, the natural carrier state occurs more frequently and persists for a longer time in brook trout than in rainbow trout (McAllister *et al*., 1993; Sadasiv, 1995; Yamamoto, 1974).

IPNV has been shown to be associated with the leukocytes of the host in which it persists, and its leukotropism has been well documented and used frequently to asses the carrier state of a population. Swanson and Gillespie (1982) investigated the ability of the virus to replicate in the peripheral leukocytes of rainbow trout and concluded that these cells harbor IPNV but did not contribute significantly to the high titers of the virus in hematopoietic organs. However, they postulated leukocytes as a very useful sample for diagnosis without lethal sampling.

Yu *et al*. (1982) reported the constant recovery of IPNV from the leukocytes of an asymptomatic 1-year-old brook trout population, and a similar virus–cell association was demonstrated by Ahne and Thomsen (1986) in adult rainbow trout after 15 months of experimental infection with several IPNV serotypes. Knott and Munro (1986) reported the presence of IPNV in leukocytes of Atlantic salmon adults,

which showed a suppressed response to mitogens. Tate *et al*. (1990) reported that the infection of leukocytes and the suppression of immune responses are involved in the establishment of the IPNV carrier state. The leukotropism of IPNV was also verified in adult rainbow trout populations by Rodríguez *et al*. (1991), who analyzed purified leukocytes from peripheral blood samples of brood stocks by flow cytometry and detected viral antigens and infective virus. After cultivation of leukocytes for 7 days or cocultivation on CHSE-214 cells, the number of fluorescent leukocytes showed high increases, suggesting multiplication of IPNV. More recently, the same authors assessed the convenience of leukocytes as a practical sample to detect IPNV carriers by either flow cytometry or polymerase chain reaction (PCR) assays (Rodríguez *et al*., 2001a). Evidences of IPNV replication in adherent head kidney leukocytes were provided by Johansen and Sommer (1995), who detected significant increases in both extra- and intracellular IPNV titers during a 7-day culture of cells from Atlantic salmon carriers.

The presence of IPNV in adults of other salmonid species of economic interest has become a question of concern for some countries. In Norway, farmed Atlantic salmon (*Salmo salar*) carrying the virus are very common (Melby *et al*., 1991); in addition, natural outbreaks of IPN in older salmonids have been reported (Smail *et al*., 1992). The disease has become a significant contributor to postsmolt mortalities, and analytical research on the viral epidemiology has two goals: how the IPNV is introduced and what risk factors promote clinical disease (Jarp *et al*., 1994). These authors concluded that the main route for introduction of the virus in salmonid farms is by horizontal transmission, but the reactivation of IPNV causing disease outbreaks after the transfer to seawater of fish is due to environmental stress, such as transport, handling, crowding, temperature, or salinity shock (Jarp *et al*., 1999). Johansen and Sommer (2001) investigated the role that acute IPNV, in comparison with an IPNV carrier condition, may play in the development of secondary diseases with other virus as infectious salmon anemia (ISA) virus. They concluded that an IPNV carrier condition at low virus titers did not influence the mortality rates after secondary infections, but when Atlantic salmons with acute IPNV were infected with ISAV, significantly fewer fish died than when fish were infected with ISAV alone. The ongoing IPNV infection seemed to provide some protection against ISA development. Similar results have been obtained in other studies of double infections involving IPNV (Rodríguez *et al*., 2001) that revealed interference and some possible immunological mechanisms. This subject needs deeper study and offers a wide field for future research.

d. Sources of IPNV The main source of IPNV is infected brood stock and survivors that become carriers and shed virus. Carrier fish can transmit the disease to other susceptible populations of fish, including their own offspring. In addition, IPNV can be introduced into a facility via virus-contaminated eggs, fry, and fingerling life stages (McAllister, 1993; Noga, 1996; Wolf, 1988).

Viable IPNV can been transported and excreted by predatory birds and mammals (Peters and Neukirch, 1986; McAllister and Owens, 1992). However, the water supply for a hatchery can harbor fish and invertebrates that are carriers of the IPNV.

C. Prevention and Control

The control of infectious diseases is very important for the productive development of the aquaculture industry. In salmonid farms, where fish are raised under intensive culture conditions, the development of control measures for viral diseases has led to the increase in fish production. In particular, the control of IPN disease has been performed by several means, including zoosanitary measures, hygienic measures, selective breeding, vaccination, and antiviral treatment.

1. Zoosanitary Measures

At present, the only truly effective method of controlling the disease caused by the IPNV in aquaculture is by prevention of exposure of susceptible fish to the virus.

In some areas where the disease is not endemic, a policy of exclusion and eradication of the virus is the best means of control, but it requires exceptionally high levels of monitoring, certification, and cooperation between farmers and responsible authorities. Consequently, fish health inspection programs have been developed to identify infected populations. The success of such fish health management programs depends on the rapid detection and identification of specific pathogens. A rapid and effective diagnosis of IPNV is essential to control the disease and the spread of the virus. Early diagnosis combined with epizootiological studies could be used to control disease by banning the movement of infected fish eggs, fingerlings, adults, and asymptomatic carriers, by intensive care of infected fish, and by selecting virus-free eggs, fry, or virus-resistant fish.

Sampling procedures for the diagnosis of disease, maintenance of the disease-free status of trout farms accredited for export purposes, and requirements for the import of ova are based on the standards recommended in the International Aquatic Animal Health Code of

the International Office for Epizooty (OIE, 2000). The control of IPN in enzootic areas can be achieved by preventing the vertical and horizontal transmission of the virus. This requires the use of eggs from fish that are free of IPNV and the rearing of fish in pathogen-free water supplies. Restriction of the movement of live fish and eggs has been applied to reduce and contain the transmission of IPNV.

The movement of infected eggs and fish within and among countries will probably continue to occur, despite attempts in several countries to avoid this type of transfer by strict fish health certification requirements.

2. Hygienic Measures

A hatchery represents a closed environment where strategies can be defined to obtain control of the virus diseases. No prophylactic or therapeutic treatments exist other than the destruction of infected stocks and disinfections of the hatchery facilities. All holding facilities and fish handling and personal equipment should be cleaned and disinfected after an outbreak occurs in a facility to avoid the risk of recontamination. Disinfection methods used to inactivate IPNV in water supplies of a hatchery include the use of ozone, ultraviolet light, or chlorine. Practical decontamination of IPNV-contaminated water supplies by ultraviolet irradiation is not likely because the virus is highly resistant to this agent. An average UV dose of 1188 ± 57 J/m^2 is required for a three-log reduction (99.9%) in the virus titer. However, IPNV is inactivated effectively in waters within 60 s when exposed to 0.1–0.2 mg/liter residual ozone, obtaining a 99.99% reduction (Øye and Rimstad, 2001).

In aquaculture production systems, the control of mortality is often achieved through the manipulation of fish density (LaPatra *et al*., 1996; Piper *et al*., 1982). Reduction of the population density of hatchery-reared rainbow trout can decrease the fish mortality in an IPN outbreak (Jørgensen and Bregnballe, 1969). This effect is a consequence of the changes in propagation dynamics of the virus and of the improvement of the immunocompetence due to the decrease of the stress on the fish (Smith *et al*., 2000).

3. Selective Breeding

Systematic breeding for increasing natural resistance in fish constitutes a potentially useful strategy for the control of infectious diseases. As a result of extensive studies over several years, a major variation in genetically determined resistance to IPN has been found among cultured rainbow trout in Japan (Okamoto *et al*., 1987). This variation

has facilitated the selection of IPN-resistant or susceptible strains and the utilization of these strains in molecular genetic research to perform linkage analysis (Okamoto *et al.*, 1993). In a study by Ozaki *et al.* (2001), QTLs for IPN resistance were identified in a segregated population of backcrosses derived from outbreeding the resistant strain of rainbow trout and susceptible strains.

4. Vaccination

An excellent review of the history and prospects of the immunoprophylaxis against IPNV has been carried out by Dorson (1988). Different strategies for developing IPNV vaccines have been evaluated since the IPNV isolation in 1960. However, the vaccines have not fulfilled the expected beneficial effects because fish at the age more susceptible to infection (fry) have still not reached a fully developed immune system (Wolf, 1988; Lillehaug, 1997). The vaccine against IPNV could be used to provide protection to fry and could be applied to eliminate the vertical transmission of IPNV from brood stock to progeny. Sano *et al.* (1981) demonstrated that adult rainbow trout injected with inactivated virus produced neutralizing antibodies.

At present, one licensed vaccine against IPNV in Atlantic salmon containing recombinant protein as an immunogen is available (Christie, 1997). Due to the lack of a good challenge model, the effect of the recombinant component has only been demonstrated so far indirectly through monitoring the antibody response (Frost *et al.*, 1998).

To be able to develop new, inexpensive, and effective vaccines for the growing aquaculture industry, more research should focus on the immune system of aquatic organisms, especially the cellular immune system (Lorenzen, 1999).

The ideal viral vaccine for IPN should fulfill the following requirements:

1. To protect the fish at early steps of its culture and to allow rapid onset of the protection. These diseases occur primarily at the fry age of fish when they are susceptible.
2. To protect against a wide variety of antigenically different strains. An optimal vaccine should contain neutralization epitopes conserved among as many strains and serotypes as possible.
3. To provide immunity of long duration and to prevent the formation of virus persistence.
4. To be administered easily. For fish farmers, oral vaccines are the ideal method of immunization.
5. To be inexpensive to produce and license.

6. For optimal protection of salmonids in seawater, vaccination should be carried out some time before sea transfer in order to give immunity sufficient time to develop and to avoid handling stress during smoltification. However, vaccination should not be carried out too early, as the degree of immunity declines with time.

a. Inactivated Vaccines Killed virus vaccines are prepared by growing virus in large amounts on cell cultures. The virus is then harvested and inactivated with formalin or β-propiolactone under conditions that ensure the retention of the immunogenic activity of the protective antigens but no virulent virus remains in the vaccine. The current strategies for an inactivated vaccine are expensive, labor intensive, and subject to the risk of the presence of noninactivated virus.

Vaccination with inactivated virus has been tested in rainbow trout given by the oral route, immersion, and injection. Protection against challenge was obtained only by injection (Dorson, 1977; Hill *et al*., 1980). However, injection of a large numbers of young fry is not practical in a hatchery situation.

Bootland *et al*. (1995) immunized adult brook trout with a high dose of inactivated IPNV, which induced a high humoral immune response with IPNV-neutralizing antibodies. However, the antibodies did not prevent infection following challenge and the immunity was often short.

b. Attenuated Vaccines Vaccination with live vaccines is, in practice, an infection with an attenuated virus strain. The virus multiplies in the host and induces an immune response by specific antibody production. The attenuated vaccines may develop by attenuation of a wild strain of virus under special culture conditions or using natural nonpathogenic strains of the virus.

IPNV can be attenuated readily by passage in cell culture. However, avirulent cell culture virus, capable of producing an active infection, has not proven to be of great value as a live vaccine. Thus, Dorson *et al*. (1978) infected fry of salmonids with an avirulent cell culture virus and virus of serotype Ab. Neither the Ab strain nor the serially passaged Ab variant conferred protection, but successful vaccination was obtained with an avirulent IPNV strain isolated from perch *(Perca fluvialis)* when this virus was delivered by immersion vaccination after hyperosmotic treatment (Leong and Fryer, 1993).

A live vaccine is not an acceptable strategy for environmental risk reasons, as avirulent virus may revert to virulence in salmonids or

other species. Also, the attenuated virus may be avirulent in the vaccinated species, but virulent in other species in the watershed.

c. Subunit Vaccines Subunit vaccines are composed of a part of the virus particle responsible for inducing protective immunity. A subunit vaccine produced by recombinant DNA techniques would provide an efficient and inexpensive alternative method for the control of this commercially important disease.

Several laboratories are involved in subunit vaccine production. Huang *et al*. (1986) have cloned portions of the A segment of the IPNV Sp serotype, which codes to VP2, the virus protein, which is the target of neutralizing antibodies. The authors synthesized the polypeptide in *Escherichia coli* for use as a subunit vaccine. Injection of this immunogen into trout resulted in protection against challenge with virulent IPNV Sp and IPNV Buhl (Manning and Leong, 1990; Manning *et al*., 1990).

However, a subunit vaccine produced by fermentation is a more realistic strategy for fish vaccine production if the protective epitopes can be identified. Epitope mapping indicates that two variable and one conserved neutralization epitopes of IPNV are localized within the third part of VP2 protein (amino acids 200–350) (Caswell-Reno *et al*., 1986; Frost *et al*., 1995). Analysis with an IPNV group A-specific neutralizing monoclonal antibody indicates that immunization with recombinant protein containing the segment (amino acids 86–210) might induce protection against all IPNV serotypes. Recombinant VP2 (rVP2), with structures that resemble these epitopes, has been expressed in *E. coli* (Frost *et al*., 1995).

Vaccination of presmolt with rVP2 included in a commercial oil/glucan-adjuvanted multivalent injectable vaccine against furunculosis, vibriosis, and coldwater vibriosis (NP-IPN) resulted in fish protection against IPN in natural outbreaks compared to fish vaccinated with the same vaccine without the IPNV component (Frost and Ness, 1997; Pettersen, 1997). This recombinant vaccine against IPNV in Atlantic salmon postsmolts has been approved for commercial use in Norway.

An additional difficulty for the development of monovalent IPNV vaccines for this species is that because the experimental IPNV challenge of Atlantic salmon does not induce mortality or IPN pathology (Sadasiv, 1995), no experimental efficacy protocols for injectable IPN vaccines exist (Leong and Fryer, 1993).

Frost and Ness (1997) demonstrated that NP-IPN vaccination of Atlantic salmon presmolts does not induce any measurable humoral response postvaccination. However, fish vaccinated with NP-IPN and

challenged with IPNV produced a rapid IPNV-specific secondary humoral immune response that correlates with an increased ability to clear the IPNV infection. Furthermore, the immunogenic bacterial components (e.g., lipopolysaccaride) and adjuvants in the multivalent vaccine formulations (NP-IPN) induce a major specific and nonspecific immune response. This probably includes the activation of macrophages and the production of cytokines such as interferon, interleukins, macrophage-activating factor, and tumor necrosis factor (Secombes *et al*., 1996).

In bacterial, yeast, piscine, and mammalian cells, Labus *et al*. (2001) expressed a truncated form of the VP2 protein of IPNV (amino acids 147–307). All four recombinant antigens were recognized by a VP2-specific mAb. Furthermore, all four recombinant preparations, when used to immunize Atlantic salmon, were capable of inducing antibodies reactive with whole IPNV in ELISA.

d. Synthetic Peptides Vaccines The use of mAbs to delineate epitopes in cloned proteins to identify neutralizing and serotypic epitopes has made it possible to develop synthetic peptides as vaccines.

Peptide vaccines offer several advantages over other vaccine types, as they are more stable, noninfectious, and simpler to produce by recombinant DNA technology or peptide synthesizers. Technology is now available for the construction of fish viral vaccines once the T and B cell epitopes recognized by fish are identified, but there is restricted by the need of a better understanding of fish response to different antigens.

e. DNA Vaccines Genetic immunization using naked DNA is the most recent approach in the design of vaccines against pathogenic microorganisms (Babiuk *et al*., 1996). This technology is based on observations that skeletal muscle cell injected with purified plasmid DNA is able to express the plasmid DNA-encoded proteins. The newly synthesized antigen can stimulate a specific immune response.

Anderson (1996) was the first to report that an intramuscular injection of plasmid DNA encoding a rhabdovirus (IHNV) G protein could induce immunity to a subsequent challenge with virulent IHNV in rainbow trout. Subsequently, DNA vaccination studies by Lorenzen and Olesen (1997) and Lorenzen *et al*. (1998) showed protective immunity in rainbow trout when challenged with viral hemorrhagic septicemia virus (VHSV). At the time of writing this review, there were no reports of DNA vaccines for IPNV.

5. Antiviral Treatment

Attempts have been made to break the viral transmission cycle by the treatment of fish or eggs with disinfectants. The virus is susceptible under *in vitro* conditions to iodophors (Amend and Pietsch, 1972), chlorine (Dorson, 1982), ethanol and methanol (Inouye *et al*., 1990), halocyamine A (Azumi *et al*., 1990), and ammonium chloride (Farias *et al*., 1988). However, IPNV is resistant to quaternary ammonium compounds, propanol and phenol (Dorson and Michel, 1987; Inouye *et al*., 1990).

Furthermore, the effect of some antiviral agents on IPNV replication has been studied. The first study using ribavirin and its derivatives to inhibit IPNV replication was carried out in 1980, with successful results *in vitro* but not *in vivo* (Jashés *et al*., 2000; Migus and Dobos, 1980; Savan and Dobos, 1980).

One of the most efficient inhibitors of IPNV replication *in vitro* was 5-ethynyl-1-β-D-ribofuranosylimidazole-carboxamide (EICAR) (Jashés *et al*., 1996). The *in vivo* antiviral effect of EICAR was evaluated in coho salmon and rainbow trout fry, infected experimentally with IPNV (Jashés *et al*., 2000). Results showed that survival of the infected fish group treated with EICAR was similar to the survival observed in the healthy control group (approximately 94%), whereas survival of the infected and untreated control fish was 56% for salmon and 28% for trout.

At the present time there is no licensed chemotherapeutic drug for the control of IPNV infections in fish, and it is likely that the costs of these compounds make their use prohibitive for aquaculture practice.

IV. Diagnostic Methods

Disease outbreaks may cause important losses to the aquaculture industry because, as viral diseases remain untreatable, control has relied upon avoidance. Commercial aquaculture facilities require a determination of the health status of fish stocks before sale or purchase. These examinations include diagnostic methods that should be applied (Desselberger, 1995; Thoesen, 1994): (i) when treatment and handling depend on diagnosis; (ii) when confirmation of the etiology of a disease provides control tools; and (iii) for epidemiological studies.

In Europe, great efforts have been made to control certain microbial diseases; in addition, regulations for zoning of free areas with respect to the noticeable diseases have been developed. All fish diseases of

potential economic importance are categorized in three lists, according to criteria outlined in Annex A of Council Directive 91/67/EEC. List I includes diseases exotic to the EU; list II comprises diseases restricted to certain locations, which are possible to control; and diseases of economic importance without possibility of eradication from a defined area are included in list III, e.g., IPN. Early diagnosis combined with epizootical studies provides a tool to control the viral disease by avoiding the movement of infected stocks among farms, different areas of a country, and different countries.

Because effective vaccines or antiviral drugs are not yet available for IPNV and other fish viruses, the improvement of rapid and sensitive methods of virus diagnosis is the most adequate approach to prevent exposure to viral infections in aquaculture facilities. Although the characteristic clinical signs of disease are frequently useful in the recognition of any particular viral disease of fish, sometimes these symptoms and gross pathology of many fish diseases are very similar (in particular IPNV and IHNV). Therefore, isolation and accurate identification of the virus should confirm the preliminary diagnosis.

Electron microscopy was the only diagnostic possibility until cell culture systems were developed in which virus multiplication can be visualized. The IPNV was the first fish virus to be cultured *in vitro* using cell tissues of trout (Wolf *et al*., 1960). Since then, the most reliable and acceptable diagnostic procedure for the viral diseases of fish is still the isolation of the causative infectious agent in tissue culture followed by serological confirmation.

Several other diagnostic methods for IPNV have been reported, including the fluorescent antibody technique, the immunostaphylococcus-protein A test (ISPA), the coagglutination test, the enzyme-linked immunosorbent assay (ELISA), immunoblots and Western blots, and immunoperoxidase phosphatase cell staining (IP). More recently, molecular probes for the detection of nucleic acids using the polymerase chain reaction have been developed and applied for the diagnosis of fish viruses.

A. Virus Isolation in Cell Cultures

The traditional method for the detection of fish viruses is based on isolation of the causative agent in susceptible cell lines and further identification by serological techniques. This method is widely accepted and still used currently as control of other diagnostic methods (Amos, 1985; Ganzhorn and LaPatra, 1994; Hill, 1976; OIE, 2000; Wolf, 1988).

In most cases of fish viral epizootics, the virus can be found in many different tissues of the affected animals with clinical signs of disease. For virus isolation, moribund fish are the best for sampling and must be alive when collected and kept on ice but not frozen, as the IPNV is stable at low temperature but a single freeze and thawing cycle could produce loss of infectivity in some strains. Samples should be processed as soon as possible but always before 48 h. In the case of small fish, several pools of 5–10 typical diseased fish should be examined. For larger fish, visceral pools could also be a good choice sampling for delivery to the laboratory, and at least 10–15 selected moribund individuals must be tested in pools of 3–5 fish each (Hill, 1976; Wolf, 1988).

For the detection of IPNV carriers, a statistically significant number of specimens must be collected. The minimum sample size for each lot must be in accordance with a plan that provides 95% confidence that infected specimens will be included in the fish sampled, assuming a minimum prevalence of infection of 2%, 5%, and so on (Ossiander and Wedemeyer, 1983). A lot is defined as "a group of fish of the same species and age that originated from the same discrete spawning population and that always have shared a common water supply" (Amos, 1985).

Samples of bacteria-free extracts at two or more dilutions should be inoculated onto duplicate susceptible cell lines derived from several fish species, such as RTG-2 (rainbow trout gonad), BF-2 (bluegill fry), or CHSE-214 (chinook salmon embryo) cells. Cells should be examined daily for cytopathic effects. If CPE does not occur after 7 days, a blind passage must be performed and observed again for another 7 days. In an interlaboratory comparative test of the EU Reference Laboratory that included 11 European laboratories, the susceptibility of five selected cell lines to three fish pathogenic viruses was studied. Results indicated that even if all viruses could be detected on all the five cell lines, the BF-2 and CHSE-214 were the most sensitive cell lines for the isolation of IPNV (Lorenzen *et al.*, 1999).

Although widely used in most virology laboratories, virus cultivation is relatively expensive and time-consuming. Many laboratories still consider it to be the most sensitive method for the diagnosis of fish virus, with a theoretical detection limit of one viral particle. However, the effectiveness of this method obviously depends on the susceptibility of the cell line (McAllister, 1997).

B. Serological Techniques for Identification

Once isolated, identification of the virus is required, which is usually performed by serological techniques (Sanz and Coll, 1992). The most commonly used serological method for virus identification is the neutralization test with specific polyclonal antisera. The neutralization test is considered a reference technique because it is specific, sensitive, and easy to interpret (Kelly and Nielsen, 1993; Lientz and Springer, 1973), although this technique is not rapid, as it requires from 7 to 21 days to obtain confirmatory results (Ahne and Thomsen, 1986).

The heterogeneity of IPNV requires that polyvalent antiserum can be used for their positive detection; however, strain-specific antisera are needed for more definitive identification (Hill *et al*., 1981). Relationships between several strains have been investigated by cross-neutralization (Hill and Way, 1995), and genetic analyses are in progress to correlate the serotypes and genotypes of aquabirnaviruses (Biering *et al*., 1997; Heppell *et al*., 1992; Lee *et al*., 1996; Novoa *et al*., 1995).

A common problem involved in the neutralization test is the viral concentration used in the assay, as well as the amount of noninfectious virus produced during replication (Nicholson and Pochebit, 1981). For these reasons, it is very difficult to standardize the antigen dose used in the test. Dopazo and Barja (2002) proposed that standardization of serological techniques would come from the use of mAbs. However, only a limited number of panels of mAbs have been employed, which cause a misdetection of new IPNV isolates (Dominguez *et al*., 1990; Vázquez-Brañas *et al*., 1994). The technology to obtain mAbs is not available in every laboratory, mainly because special skills are required. In order to obtain real standardization of the procedure, a unique panel of mAbs should be available commercially for both detection and identification of the specific serotype of a new IPNV isolate.

1. Immunofluorescence

Immunofluorescence has been used to study fish viruses since 1972 (Vestergard-Jorgensen and Meyling, 1972) and is widely accepted for the detection and identification of IPNV in cell cultures. Both direct (DFAT) and indirect (IFAT) fluorescent antibody tests can be used, but the IFAT is usually preferred because only one or two antispecies conjugates against different primary antisera are required, and, in addition, IFAT shows a higher sensitivity compared to DFAT (Gardner, 1986).

Piper *et al.* (1973) were the first to describe the one-step growth curve of IPNV by the DFAT technique, detecting viral antigen at 3–4 h postinfection and 75% of the cells containing antigen in the cytoplasm at 9 to 11 h postinfection. Later, Tu *et al.* (1974) developed an IFAT assay for the diagnosis of IPNV infections, defining the conditions for an optimal use in cell cultures. Results obtained by these authors allowed the count of the number of infected cells in coverslip cultures, being confirmed many years later, using the flow cytometry technique (Rodriguez *et al.*, 1995). Cells should ideally be incubated for 10–12 h postinfection to detect the maximum number of stained cells in the first cycle of infection. Prolonged incubation (24 h postinfection) provides the observation of cells infected in the second cycle of infections and may be a more adequate and useful staining time in routine laboratory practice. However, in several emergency situations, IFAT can be performed after only 7–8 h incubation (Rodriguez *et al.*, 1995; Tu *et al.*, 1974).

The IFAT assay has become a universally accepted and standardized method for inspections and certifications of fish in the European and United States programs for fish health regulations (EEC, 1996; OIE, 2000). This technique possesses several advantages in comparison with conventional isolation techniques, such as rapidity, specificity, sensitivity, and reproducibility. Nevertheless, IFAT is generally labor-intensive and requires a highly trained staff for reading the stained samples (Rodriguez *et al.*, 2001).

2. Immunoperoxidase Stain

The IP stain assay is similar to IFAT but uses horseradish peroxidase or alkaline phosphatase-labeled antibodies instead of FITC-labeled antibodies. For identification of IPNV and other fish viruses in cell cultures, the IP stain was first proposed by Faisal and Ahne (1980), who compared this stain with the IFAT and reported a higher sensitivity degree of the IP stain because the viral antigens were detectable earlier. Ahne (1981), comparing several serological techniques, concluded that the IP assay could be used for diagnostic purposes on the basis of its simplicity, rapidity, high specificity, and permanent record of preparations. The IP stain has been used frequently in immunohistochemistry to study the pathogenesis of IPNV and other fish viruses (Drolet *et al.*, 1993; Kim *et al.*, 1994). In a comparative study of diagnostic methods for IPNV, Rodríguez *et al.* (2001b), reported that the immunoperoxidase test allowed early detection of IPN viral antigens at high multiplicity of infection, but had lower sensitivity than immunofluorescence, as required infective titers of $1\times10^{3.5}$ $TCID_{50}$/ml for

67% positive detections versus the 80% of positive detection obtained by immunofluorescence test at 1×10^2 $TCID_{50}$/ml.

3. ISPA Assay

This method was first used by Bragg and Combrink (1987) for the specific detection of IPNV in a wide survey of diseased rainbow trout in South Africa. The assay is based on the use of protein A of *Staphylococcus aureus* that nonspecifically binds the Fc fragments of IgG of immune sera (Lancz and Specter, 1986). The test uses formalin-fixed *S. aureus* cells sensitized with polyclonal rabbit anti-IPNV antiserum that, when mixed with virus grown in cell culture, binds specifically to the infected cells and the attached bacteria could be clearly visualized.

A variant of the ISPA assay, the coagglutination test, is based on the same principle, but it is more simple, using directly the antibody-coated cells to be mixed with samples containing IPNV; the antibody binds specifically to the virus and causes the bacterial cells to agglutinate. Kimura *et al*. (1984) reported the successful use of the coagglutination test for detection and serological typing of many different strains of IPNV from several countries.

The advantages of these methods are their relatively simplicity and the fact that they do not require special equipment and can be performed in the field. On the contrary, the low sensitivity of the methods, the need of high viral titers, and the fact that they are not suitable for use in subclinical infections or for detection of fish carriers are the main shortcomings of these methods.

4. Enzyme-Linked Immunosorbent Assay

The ELISA is a widely accepted technique used in health programs for virus control in the EU (OIE, 2000), both the variant of the sandwich or capture ELISA and the direct ELISA. The first step of the technique consists of adsorption of an antivirus antiserum (or the purified fraction IgG) to a solid phase; later, labeled antibodies and the substrate are added and the reaction (hydrolysis of the substrate) is visible by a color change.

The ELISA technique can detect and identify IPNV in cell cultures using polyclonal or monoclonal antibodies. Nicholson and Caswell (1982) and Dixon and Hill (1983) reported the specificity and rapidity of ELISA for IPNV detection and serotyping. Hattori *et al*. (1984) applied the technique for the *in vitro* and *in vivo* detection of IPNV serotype VR299 using cell cultures and rainbow trout fry infected experimentally, respectively. The ELISA technique is a rapid, easily automated, and reliable method for the diagnosis of IPNV, which could be detected at 10^4TCID_{50}/ml in cell culture fluid (Dominguez *et al*.,

1990; Rodak *et al.*, 1988). Monoclonal antibodies could increase the specificity and sensitivity of the assay, improving the ability to detect virus in fish tissues without background.

5. Immunoblots (Dot Blots)

The immunoblot assay or dot blot is a variation of the ELISA technique. Instead of binding antigen to wells, antigen is bound directly to a nitrocellulose or nylon membrane and is detected with labeled primary antibody or indirectly with labeled secondary antibody. McAllister and Schill (1986) developed this assay for salmonid fish viruses and considered it a rapid, sensitive, and inexpensive technique for virus identification in cell culture fluids. However, the immunoblot reactivity is showed at titers of 10^5–10^6 $TCID_{50}$/ml, which, in comparison with other methods, represents a very low sensitivity. The principal advantages of the dot blot are its technical simplicity, easy interpretation, and that it is not time-consuming.

6. Flow Cytometry

The use of flow cytometry allows the detection of cells stained with a fluorescent dye and to relate this parameter to others of the individual stained cells in an environmental population (Brussaard *et al.*, 2000). Flow cytomety also offers great potential for fish pathology investigations, but so far few studies have been devoted to pathogenic and immunopathological processes (Chilmonczyk and Monge, 1999), mainly due to the need to use sophisticated equipment not always available.

In the field of fish virology, an indirect immunofluorescent stain applied to several types of infected cells and monitored by flow cytometry has been developed for IPNV detection and virus–cell interaction studies (Alonso *et al.*, 1999b; Pérez *et al.*, 1994; Perez-Prieto *et al.*, 2001; Rodríguez *et al.*, 1991, 1992, 1995). The assay allows a quick reading of 2000 cells per second, examination of a large number of samples, is especially accurate for the detection of IPNV carriers, with a level of sensitivity comparable to that of virus isolation in cell culture, and is less time-consuming. In addition, it has been applied successfully in nonlethal sampling of sperm cells (Rodríguez *et al.*, 1992) or blood leukocytes (Rodríguez *et al.*, 1991, 2001).

C. Molecular Diagnostic Techniques

The concept of molecular techniques of diagnosis is generally associated with DNA-based techniques, mainly nucleic acid hybridization and the polymerase chain reaction, which were the first used for

diagnostics of fish viruses (Christie *et al*., 1988; Rodak *et al*., 1988). Nevertheless, other techniques involving detection and characterization of the viral genome or polypeptides must also be considered.

A common practice is the use of a molecular technique for identification of a previously isolated virus. Some authors have applied those techniques for detection and identification of the virus in inoculated cell culture before CPE would be visualized, which implies that isolation is not essential (Dopazo *et al*., 1994). Detection and identification of the virus directly in fish tissues have the advantage of avoiding the need for viral isolation. However, it has several disadvantages, such as the optimization of the viral nucleic acid extraction from the fish tissues and the elimination of inhibitors that interfere with the molecular technique (Wiedbrauk and Farkas, 1995).

1. Electropherotypes Analysis and Fingerprinting

Separation of the RNA and polypeptide components of the virus has been proven to be an important tool for the diagnosis of IPNV isolates. The bisegmented dsRNA that constitutes the viral genome of IPNV is very stable and easily identified by SDS–PAGE. The electropherotypes (EFTs) of genomic segments and polypeptides, i.e., their mobility in SDS–PAGE, have been widely used to identify isolates of IPNV (Hsu *et al*., 1989; Novoa *et al*., 1993a; Jung *et al*., 1999) for comparison of different strains of IPNV and for typing of new isolates (Cutrin *et al*., 2000; Ganga *et al*., 1994; Hedrick *et al*., 1985; MacDonald and Gower, 1981; MacDonald *et al*., 1983; Novoa *et al*., 1993a, 1993b; Sohn *et al*., 1995).

Considering the economic and ecological importance of the IPNV in salmonids, characterization of new isolates as part of management programs is essential to determine the diversity of viral isolates and to establish the geographical relationships. Thus, the determination of genomic variation (electropherotypes) is simpler and more reliable for the classification of viruses than serological techniques, as it is not influenced by uncontrolled factors (Dopazo, 1991). Moreover, this technique has been used in epizootiological studies to identify the possible origin of a certain isolate (Cutrin *et al*., 2000; Espinoza *et al*., 1985; Hsu *et al*., 1989, 1993; Novoa *et al*., 1993a).

A method based on RNA fingerprinting was reported by Hsu *et al*. (1995) to characterize the genome heterogeneity among IPNV isolates. The technique involves a two-dimensional gel electrophoresis of ^{32}P-labeled viral RNA subjected to RNase treatment in the presence of tRNA used as a carrier. Later, the gel is exposed to X-ray film, and the pattern of oligonucleotide spots is analyzed by a computer.

Unfortunately, this technique has not been compared to other studies perhaps because it is cumbersome and needs special skills.

2. Restriction Fragment Length Polymorphism

The analysis of restriction fragment length polymorphism (RFLP) can be applied for the rapid typing of fish viruses, being an alternative method to EFTs or to the serological techniques. Heppell *et al*. (1992) analyzed the RFLPs of strains of 10 IPNV serotypes based on a 359-bp cDNA fragment corresponding to the highly variable NS coding region. The results obtained allowed them to establish three major groups that almost corresponded to the traditional serotypes A1, A2, and A3, and 10 subgroups poorly correlated with serotypes.

Other authors trying to establish a correlation between genotyping and serotyping applied RFLP analysis to cDNA fragments obtained from the hypervariable VP2 coding region (Biering *et al*., 1997; Novoa *et al*., 1995a, 1995b), but they did not find a close correlation. Better results were obtained by Lee *et al*. (1996), who performed RFLPs with an 1180-bp fragment representing most of the VP2 region. They found that genogrouping was identical to serological classification with VP2-specific mAbs.

Although the RFLP procedure is practical for typing large numbers of strains, it can be used neither in epidemiological studies nor in identification of new isolates because the lack of standardization of the assay.

3. Nucleic Acid Sequencing and Hybridization

Nucleic acid sequencing has been used for genogrouping birnaviruses and comparison of their sequences with the serological group (Heppell *et al*., 1993, 1995; Hosono *et al*., 1996). Other authors have employed sequencing of the viral genome for typing new isolates of IPNV (Havarstein *et al*., 1990; Pryde *et al*., 1993). However, although it is a useful method to group aquatic birnaviruses, the technique is time-consuming and relatively expensive.

RNA–DNA dot-blot hybridization constitutes a useful tool for detecting and identifying IPNV in inoculated cell cultures, even before CPE can be visualized. Nevertheless, this technique is poorly efficient in the detection of the viral genome directly in IPNV-infected fish tissues (Dopazo *et al*., 1994), and a sensitivity limit of direct hybridization of 10^4 to 10^5 molecules must be considered (Desselberger, 1995).

This method has been applied to the identification of IPNV strains using several oligonucleotide probes (Christie *et al*., 1988a; Dopazo

et al., 1994; Rimstad *et al*., 1990a). Biering and Berg (1996) have used this technique for *in situ* hybridization (ISH) and have compared it to immunohistochemistry (IHC). Although ISH did not appear to be more sensitive than IHC, background staining was absent and the labeling was localized easily in the cytoplasm of the infected cells.

4. Polymerase Chain Reaction

The polymerase chain reaction is a powerful technique used to amplify specific regions of DNA (PCR) or RNA (RT-PCR) to easily detectable levels on agarose gels or with nucleic acid probes. This technique uses two primers that span the nucleic acid sequence of interest and a thermostable polymerase to exponentially increase the amount of nucleic acid through repeated cycles of synthesis (Winton, 1991). Although it is not yet used for diagnosis purposes in fish virology, the use of RT-PCR for detecting aquatic birnaviruses in infected cell cultures is increasing (Alonso *et al*., 1999a; Blake *et al*., 1995; Lopez-Lastra *et al*., 1994; McAllister *et al*., 1991; Pryde *et al*., 1993). Several authors have demonstrated that RT-PCR is a rapid, reliable, sensitive, and convenient method of diagnosis (Novoa *et al*., 1995; Rodriguez *et al*., 2001; Wang *et al*., 1997; Williams *et al*., 1999).

Most of the PCR assays described have been developed for detecting the pVP2 gene of IPNV, as it is the major capsid protein and is probably the best target for amplification and detection. Selection of specific primers has been possible, as the nucleotide sequence of genome segment A has been determined for the Jasper IPNV strain (Duncan and Dobos, 1986), the Sp strain (Mason, 1992), and the N1 strain (Havarstein *et al*., 1990). The sequence of segment B was also established for Jasper and Sp strains (Duncan *et al*., 1991).

The high sensitivity of this method for detection of IPNV has been demonstrated. Thus, Rimstad *et al*. (1990), using a double-nested PCR, obtained a sensitivity of 10 pg (about 1.3×10^6 particles/reaction) for the detection of purified RNA from salmonid isolates. Detection levels of 1 pg were reported by Lopez-Lastra *et al*. (1994) in a study where the authors also reported that nested PCR detected asymptomatic carriers in field samples. Coupling of a nested PCR amplification assay, using the first round amplification products as templates, could enhance the sensitivity.

However, several researchers have used the PCR technique both to identify viruses isolated in cell cultures and to confirm the etiology of isolates identified by the neutralization assay (Alonso *et al*., 1999; Jung *et al*., 1999; Rodriguez *et al*., 1995, 1997). Furthermore, McAllister *et al*. (1991) demonstrated the potential use of a single RT-PCR

assay for the simultaneous detection of different fish viruses (IPNV, VHSV, and IHNV). Using this technique, Williams *et al.* (1999) reported sensitivities of 100, 1, and 32 $TCID_{50}$/ml for the simultaneous detection of IPNV, IHNV, and VSHV, respectively.

Heppell *et al.* (1992) were the first to apply the PCR assay to the study of genomic variations between the IPNV strains. They designed a set of primers for the amplification of a 359-bp cDNA fragment of the viral genome, a region that covers the C-terminal part of VP2 and the N-terminal part of the NS protein. Fragments amplified from 37 strains were digested with several restriction enzymes, and analysis of the restriction patterns showed that IPNV strains can be divided into three major groups, corresponding approximately to serotypes A1, A2, and A3 and 10 subgroups that do not correlate with the serotyping of the strains. Pryde *et al.* (1993) also applied the PCR technique for genomic analyses of several IPNV serotypes and for identifying specific strains of aquatic birnaviruses. They described a PCR assay to amplified cDNA encoding the serotype-specific epitope of the VP2 gene of three strains of IPNV isolated from salmonids. Primers for the reaction were designed from two conserved regions flanking the variable segment of the IPNV N1 strain VP2 gene.

Once the reliability of the PCR assay as a diagnostic tool was proven, several studies were involved in testing protocols for avoiding viral amplification by cell cultures. Blake *et al.* (1995) proposed a PCR assay with a primer set that amplified a 173-bp segment of cDNA within the genomic region encoding the VP3 protein. The authors described it as a rapid (24–48 h) diagnostic test for routinely detecting aquatic birnaviruses directly in fish tissue samples at a level of accuracy and sensitivity comparable to the virus isolation procedure in cell culture. The same protocol was applied successfully to kidney homogenates of turbot (*Scophthalmus maximus*) infected experimentally with a birnavirus strain originally isolated from the same species (Novoa *et al.*, 1995).

More recently, RT-PCR has been used for the study of viral interference in IPNV–IHNV coinfections in salmonids. Alonso *et al.* (1999) demonstrated that the PCR assay is a suitable technique for identifying dual viral infections; the IPNV was always detected but the amplification of IHNV RNA was not achieved as readily. Pérez-Prieto *et al.* (2001) detected RNA of IPNV in leukocytes from gilt-head sea bream (*Sparus aurata*) infected experimentally with a virus strain of an aquatic birnavirus, serologically similar to the Sp strain. Later, these authors developed nested PCR assays that improve the sensitivity for

nonlethal detection of the virus directly from leukocytes of carrier rainbow trout (Pérez-Prieto *et al*., 2001).

In short, the PCR technique proved to be a promising procedure for the diagnosis of IPNV and other fish viruses. The technique shows at least the same sensitivity as the isolation in cell cultures, and shorter times are required for diagnosis in a survey study. The technique can be applied for identification of a previously isolated virus or even infected monolayers prior to visualization of CPE. It can also be applied to detect the virus directly in tissues from infected fish.

Moreover, PCR can detect the virus even in concentrations below the detection limit of cell culture and ensures that the virus will be detected even if susceptible cells are not available or when the sample size is too small for cell culture (Dopazo and Barja, 2002). However, several studies established that positive PCR results cannot be accepted as conclusive diagnosis on the basis of the following reasons: (i) it does not ensure detection of the infective viruses presence, but may detect naked noninfective viral nucleic acids (Alonso *et al*., 1999; Hiney, 2000); (ii) it becomes even more complicated when the possibility of prior vaccination against IPNV is considered (Leong and Fryer, 1993); and (iii) in respect to application of a fish health policy, DNA–molecular diagnostic techniques must be seen in the context of infectious disease epizootiology, taking into account the concept of disease causation (Bernoth, 1999).

As mentioned earlier one of the main advantages of PCR is the sensitivity of the method, which is theoretically capable of detecting a single target molecule (Clewely, 1989), although when the target is RNA, reverse transcription is the critical step influencing the sensitivity of the PCR (Byrne *et al*., 1988). The sensitivity for detection of IPNV has been proven repeatedly to be as high as 15 fg to 10 pg (Lopez-Lastra *et al*., 1994; Rimstad *et al*., 1990; Wang *et al*., 1997). Therefore, this high sensitivity carries serious disadvantages, as even negligible amounts of contaminating nucleic acids can produce false positives. Other critical steps influencing the effectiveness of RT-PCR include the following: (i) the procedure of extraction of viral RNA; (ii) the selection of adequate primers in order to ensure detection of the required IPNV serotype; and (iii) the choice of the protocols and kits for RT-PCR.

5. Comparative Evaluation and Conclusions

Despite the large number of articles describing the outcome or advantage of any of the mentioned methods for IPNV detection, only

a few focus on comparative analyses (Anhe, 1981; McAllister *et al.*, 1991; Novoa *et al.*, 1995; Sanz and Coll, 1992).

More recently, Rodríguez *et al.* (2001) evaluated comparatively six diagnostic methods for the detection of IPNV using 83 wild isolates, 3 reference strains, and the seroneutralization assay as the control method. The results obtained (Table I) showed that only RT-PCR tests had a similar sensitivity of detection to seroneutralization at the lowest infective titers tested (1×10^1 $TCID_{50}$/ml) and was applied successfully for diagnosing all the 83 IPNV isolates. At a viral infective titer of 1×10^2 $TCID_{50}$/ml, 80% of the IPNV tested showed positive detection by the use of flow cytometry test, whereas the immunofluorescent test showed a relative percentage of positive detection of 26.7% that increased to 100% when 1×10^3 $TCID_{50}$/ml IPNV infective titer was assayed. Lower sensitivity exhibited the immunoperoxidase, immunodot blot, and ISPA techniques, which need viral titers as high as 10^4, 10^5, and 10^7 $TCID_{50}$/ml, respectively, to achieve 100% of positive detection. The most accurate, rapid, and sensitive methods to identify IPNV were RT-PCR and flow cytometry assays, which may be applied even to detect carrier fish.

TABLE I

COMPARISON AMONG THE SIX DETECTION METHODS TESTED AND THE NEUTRALIZATION ASSAY FOR DETECTION OF IPNV AT SEVERAL INFECTIVE TITERS.[a]

Infective titer $\log_{10}$ $TCID_{50}$/ml	No. of samples with positive detection using neutralization assay	Technique tested[b]					
		IFA	FC	IP	IDB	ISPA	RT-PCR
1	5	0	0	0	0	0	100
2	15	26.7	80	0	0	0	100
3	9	100	100	22.2	0	0	100
4	25	100	100	100	92	0	100
5	7	100	100	100	100	71.4	100
6	14	100	100	100	100	71.4	100
7	3	100	100	100	100	100	100
8	5	100	100	100	100	100	100

[a] Reprinted from Rodríguez, S. *et al.* (2001). Assay for the detection of IPNV in fish. *J. Virol. Methods* **97:** 30, with permission from Elsevier Science.

[b] No. of samples with positive detection of IPNV using the tested method/no. of samples with positive detection of IPNV using the neutralization assay × 100. IFA, immunofluorescence assay; FC, flow cytometry; IP, immunoperoxidase test; IDB, immunoblot test; ISPA, immunostaphylococcus-protein A test; RT-PCR, reverse transcriptase polymerase chain reaction assay.

V. Future Research

Disease outbreaks of IPNV has been considered characteristic of young salmonid fish at ages below 6 months. However, recent and increasing detection of IPNV in reared adult salmon offers an important current issue to research, supporting the virus as an interesting pathogen with which to work.

Much is still to be learned in terms of the mechanisms by which IPNV is able to produce disease in fish and in terms of host defense and how it can be manipulated. Future research should be addressed to understand the following.

1. The establishment and maintenance of the carrier state. A significant component of viral persistence is evasion of immunological surveillance, and there is strong indication that studies of virus–macrophage interactions may be pivotal in understanding how viruses can use a strategy favoring persistence.
2. The role of defective interfering particles in viral pathogenesis *in vitro*, evaluating whether these particles can confer any prophylactic advantage to the host in an artificially induced situation, and whether defective interfering particles do alter the course of a natural viral infection.
3. The fish immune response to viral infections, from which IPNV would be a useful model. In this way, cytokines and cytokine genes are the target molecules for studies of the fish immune response, elucidating where these molecules are expressed, what factors affect expression, and what is the activity of the proteins they encode.
4. The mechanisms of interference in double infections of IPNV and other fish viruses as IHNV or ISA and the role of interferon or other immune responses.
5. New approaches to the development of vaccines and the potential use of cytokines as immunostimulants in fish.

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ADVANCES IN VIRUS RESEARCH, VOL 62

STRUCTURES OF PICORNA-LIKE PLANT VIRUSES: IMPLICATIONS AND APPLICATIONS

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I. Introduction

Plant picorna-like viruses, which include *Como* and *Nepo* genera of the Comoviridae, share similarities in structure, genome organization, and replication strategy with mammalian picornaviruses. Structural studies of comoviruses and a nepovirus contribute to the understanding of icosahedral virus structures, the development of novel methods for investigating macromolecular assemblies, and the infrastructure for virus-based bio- and nanotechnology. Notable advances coming from this work include (1) the definition of the asymmetric unit for picorna-type capsids; (2) the first view of a partial viral genome conforming to icosahedral symmetry; (3) the pioneering synthesis of electron microscopy and crystallography to investigate complex macromolecular assemblies; (4) the evolutionary links in picorna and

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picorna-like viruses; and (5) the basis for the exploitation of plant viruses for bio- and nanotechnology.

II. Background

Comoviruses, which exemplify plant picorna-like viruses, are a group of nonenveloped, icosahedral plant viruses in the Comoviridae, which also includes nepoviruses and fabaviruses (Wellink *et al*., 2000). The genomes of comoviruses are comprised by two segments of single-stranded, positive-sense RNA. The large RNA, RNA1, encodes the virus replication machinery, and the smaller RNA2 encodes the two capsid proteins and a movement protein (Lomonossoff and Johnson, 1991). One of the capsid proteins, the S subunit of about 23 kDa, folds into a jelly roll β sandwich, and the other capsid protein, the L subunit of about 41 kDa, folds into two jelly roll β sandwiches. Sixty copies of the capsid proteins form the virus capsid in a $P = 3$ symmetry (Fig. 1). Cowpea mosaic virus (CPMV) is the type member of the group and shows the properties displayed in Table I.

Based on structures and genome replication strategies, comoviruses are thought to be related to mammalian picornaviruses and insect picorna-like viruses in evolution (Fig. 2A). The two segments of the comovirus genome are encapsidated separately in isometric particles, one containing RNA1 (bottom component) and the other containing RNA2 (middle component). Both components are required for the infection (Fig. 1). Due to the different RNA content, the particles sediment differently, forming bands at different densities. The sedimentation of CPMV particles in a CsCl gradient is illustrated in Fig. 2B.

There are many members in the *comovirus* family (Wellink *et al*., 2000). The family is composed of two subtypes based on sequence alignment: one is represented by CPMV and the other by bean pod mottle virus (BPMV) (Chen and Bruening, 1992). Two other members of the family are well studied: red clover mottle virus (RCMV) in Lapchic *et al*. (1998) and references therein and cowpea severe mosaic virus (CPSMV) in Chen and Bruening (1992) and references therein. Crystal structures of three comoviruses, CPMV, BPMV, and RCMV, were determined and refined to near atomic resolution (Chen *et al*., 1989; Lin *et al*., 1999, 2000, 2003; Stauffacher *et al*., 1987). The structure of a nepovirus, tobacco ringspot virus (TRSV), was also determined and shown to share many features with comoviruses (Chandrasekar and Johnson, 1998).

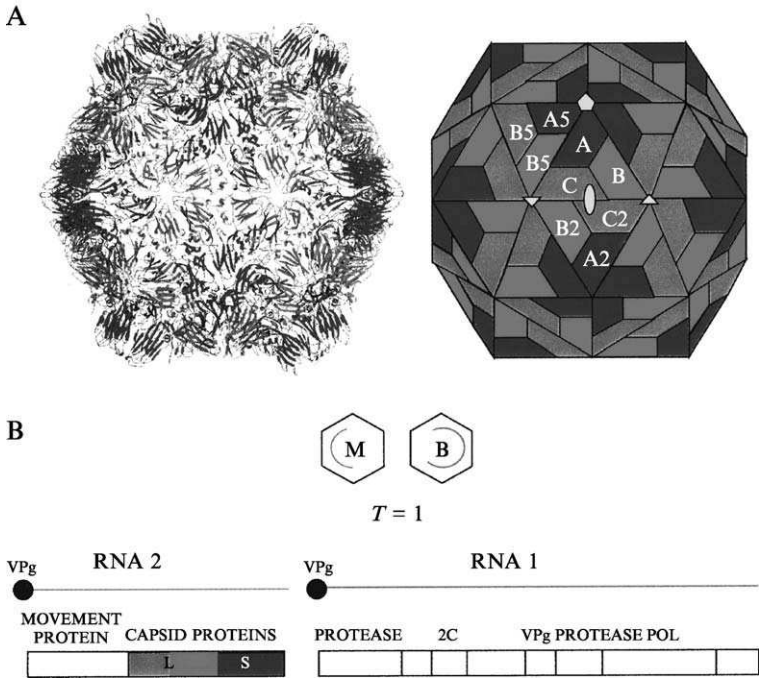
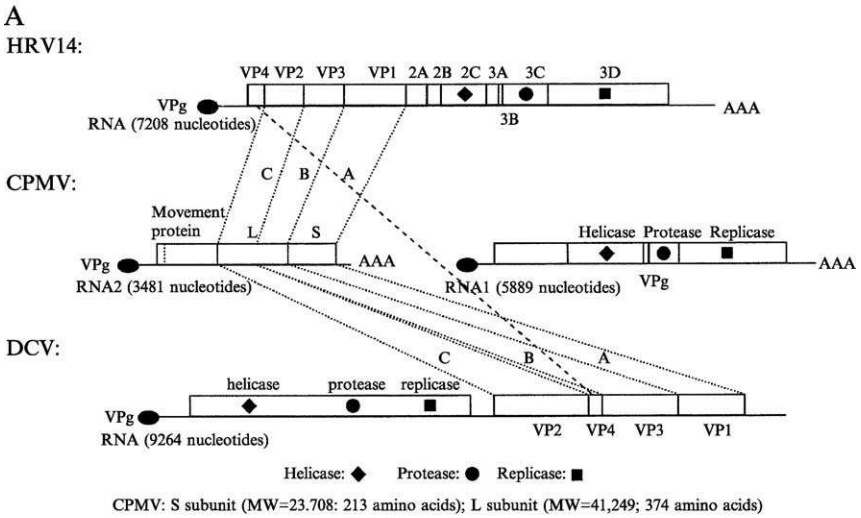


FIG 1. Schematic presentation of CPMV capsid and genome organization. (A) Ribbon (left) and schematic (right) diagrams of the virus capsid. Sixty copies of three types (labeled A, B, and C in the asymmetric unit) of jelly roll β sandwich domains comprise the viral capsid in a $T = 1$ icosahedral symmetry. Because the three domains in the asymmetric unit are arranged in a similar surface lattice to $T = 3$ viruses, except they are of different polypeptide sequences, it is generally described as in pseudo $T = 3$ or $P = 3$ symmetry. The oval represents the twofold axis, the pentagon represents the fivefold axis, and triangles are for threefold axes. S subunits occupy A positions around the fivefold axis; the two domains of the L subunit occupy the B and C positions. The quaternary interactions at A/B5 and C/C2 interfaces are pseudoequivalent. The unique intersubunit interfaces are A/B5, C/B5, A/C, A/A5, A/B, B/C, C/B2, and C/C2, which are listed in Table II with the buried surface area at each interface. (B) Two RNA molecules comprise the viral genome, and each RNA molecule is encapsidated separately. A VPg (genome-linked protein) is attached at the 5′ end of each RNA molecule. RNA1 encodes the viral replication machinery, whereas RNA2 encodes a movement protein and two capsid proteins, L and S. The L protein folds into B (red) and C (in green) domains, whereas the S protein folds into the A domain (in blue). (See Color Insert.)



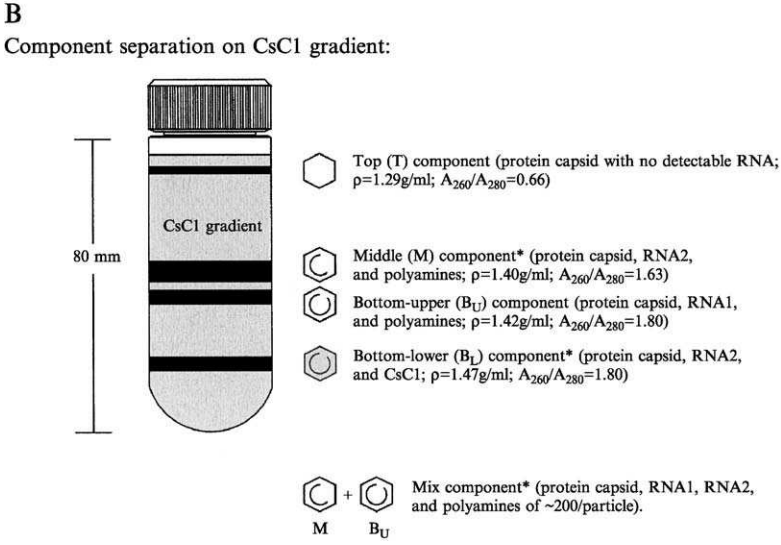


FIG 2. (A) Genome alignment of human rhinovirus 14 (HRV14), cowpea mosaic virus (CPMV), and *Drosophila* C virus (DCV), which is a picorna-like insect virus (Johnson and Christian, 1998). HRV14 is monopartite, and CPMV is bipartite. DCV, although being monopartite, is dicistronic. The genome of CPMV can be aligned with both HRV14 and DCV, as the order of the two CPMV RNA segments is arbitrary. RNA1 of CPMV encodes the virus replication machinery, whereas RNA2 encodes a movement protein and two capsid proteins, L and S subunits. The L subunit of CPMV is aligned with the VP2 and VP3 proteins of HRV14 and DCV, and the S subunit is aligned with VP1 proteins of HRV14 and DCV. The alignment of HRV14 and DCV requires a reordering of coding

TABLE I
PROPERTIES OF CPMV

RNA					
		No. of Nucleotides	M.W.		
RNA 1		5889	2.02×10^6		
RNA 2		3481	1.22×10^6		
Capsid proteins					
		No. of Amino Acids	M.W.		
L subunit		374	41,249		
S subunit		213	23,708		
Virus Components					
	%RNA	RNA	M.W.	S	Buoyant Density (g/cc)
Top	0	None	3.94×10^6	58	1.297
M	24	RNA2	5.16×10^6	98	1.402
B_U	34	RNA1	5.98×10^6	118	1.422
B_L	34	RNA1	5.98×10^6	N/A	1.470

III. COWPEA MOSAIC VIRUS (CPMV): THE TYPE MEMBER OF *COMOVIRUSES*

A. Crystal Structures

The initial crystal structure of CPMV was determined to 3.5 Å resolution (Stauffacher *et al*., 1987) and later extended to 2.8 Å resolution (Lin *et al*., 1999). Three components, MIX, M, and B_L were used in the structural studies (Fig. 2B).

sequences of the coat proteins and viral replication machinery. Also of interest, the coding sequence of VP4 is at the 5′ end of the VP2 coding sequence in HRV14, but at the 3′ end of the VP2 sequence in DCV (Tate *et al*., 1999). (B) CPMV component separation on the CsCl gradient. The two RNA molecules of the CPMV genome are encapsidated separately in different virus particles, which can be separated on a CsCl gradient. The top component, which contains no RNA, has the least buoyant density and is at the top of the gradient. The middle (M) component contains the smaller RNA2 and the bottom component contains RNA1. Due to the displacement of endogenous polyamine by Cs^+, the bottom component is further separated into bottom–upper (B_U) and bottom–lower (B_L) components. The small separation of M and B_U components often results in a preparation of a mixture of the two, which is termed the MIX component. From Lin *et al*. (1999).

1. Particle Organization

The first structure of CPMV was determined with crystals containing approximately a 1:1 mixture of the B_U and M components (Fig. 2B), which were referred to as MIX. The overall particle shape of CPMV is shown in Fig. 3 with a 12-Å-thick equatorial cross section of electron density. The particle has protrusions at the icosahedral fivefold and threefold symmetry axes and a valley at the icosahedral twofold axes. Figure 4 shows the relationship between tertiary and quaternary structures of the proteins forming the particle. Figure 4B illustrates the folds of the individual domains corresponding to the trapezoids. The three domains are distributed in two polypeptide chains. The large (L) protein is composed of 374 amino acids and consists of the C domain at the amino terminus and the B5 domain at the carboxyl terminus (see Fig. 4 for subunit nomenclature). The small (S) protein is composed of 213 amino acids and corresponds to the A domain.

2. Domain Structure Comparison

The individual domains of the L and S proteins occupy spatially equivalent positions to those found in $T = 3$ viruses formed by a single gene product. The β sandwich folds of the individual domains in CPMV are

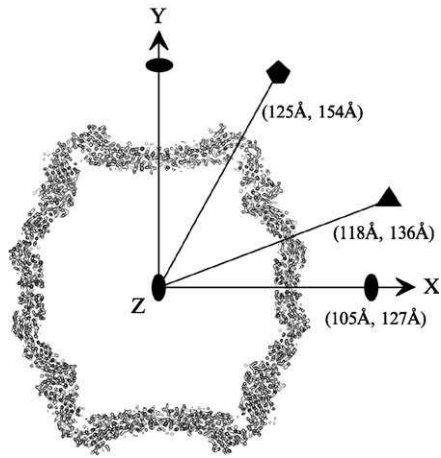


FIG 3. An equatorial cross section (12 Å in thickness) of the averaged electron density of the CPMV capsid structure. The particle has protrusions at the icosahedral fivefold and threefold symmetry axes and a valley at the icosahedral two-fold axis. The internal and external radii of the particle along each symmetry axis are indicated. From Lin *et al*. (1999).

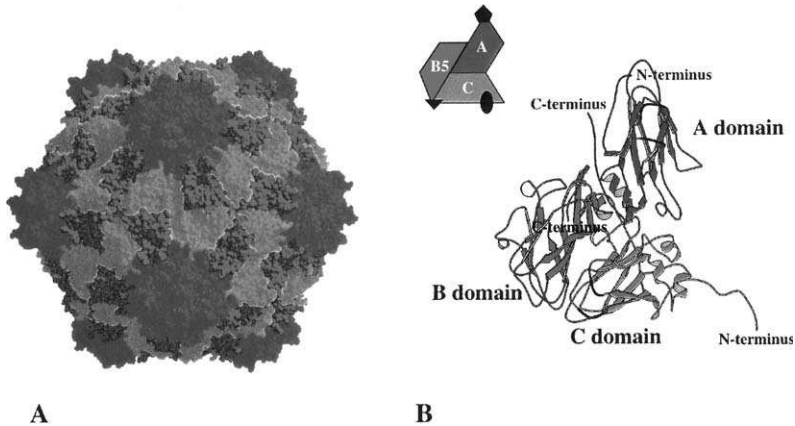


FIG 4. The structure of the viral capsid and the icosahedral asymmetric unit of CPMV. (A) A space-filling drawing of the CPMV capsid. (B) A ribbon diagram of the three β sandwich domains that comprise the icosahedral asymmetric unit. N termini of the S and L subunits are in the interior and C termini are in the exterior. All three domains are variants of canonical jelly roll β sandwiches. (Upper left) A schematic diagram of the asymmetric unit with icosahedral symmetry axes. The A domains surround the fivefold axes, and the B and C domains alternate around the threefold axis. Adapted from Lin *et al*. (1999). (See Color Insert.)

topologically identical and structurally similar. The three-dimensional structures were superimposed on each other using only the Cα atoms of the β strands, and the corresponding sequences were compared (Fig. 5). Despite the complete lack of sequence identity, the basic β sandwich folds are conserved in all three domains. The wedge-shaped β sandwich structures are approximately 50 Å long, 20 Å across at the narrow end, 30 Å at the wide end, and 20 to 30 Å in thickness. The C and B5 domains are connected by 10 residues that attach the βI strand in the C domain with the βB strand in the B5 domain. The connector is internal at the RNA interface and links the C and B5 domains with nearly the minimum number of residues required to cover the distance between the two strand termini. The small subunit (A domain) deviates most from the canonical β sandwich fold. The insert between the βC and βD strands is extended and creates two additional strands on top of the sandwich to make it a 10-stranded sandwich.

3. Quaternary Interactions

It is plausible that the picorna-type capsid of CPMV evolved from a $T = 3$ virus in which the subunit gene was triplicated, allowing independent evolution of the sequence and structure of the individual

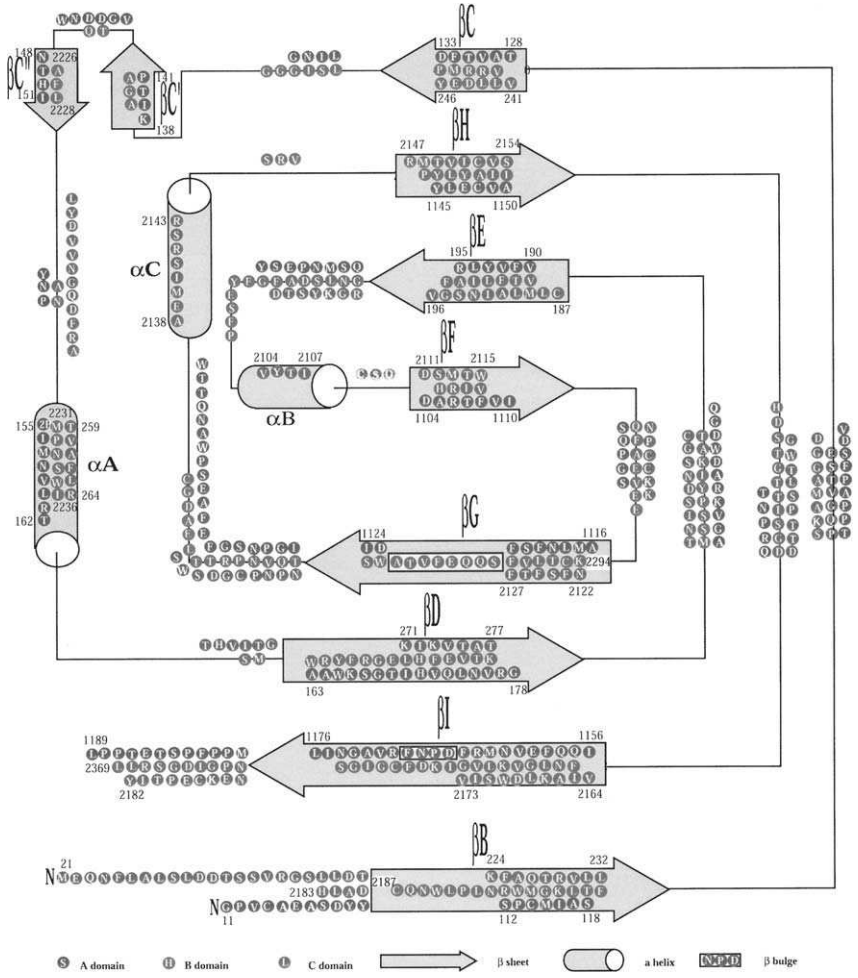


FIG 5. Structure-based alignment of three domains of CPMV capsid protein sequences. Sequence alignment is based on the structural superposition. Strands in β sandwiches are aligned as indicated. Both A and B domains contain additional, although structurally different, β strands, βC' and βC" strands, after the βC strand. In the A domain, the βC' and βC" strands lie on top of the βB and βC strands, forming a 10-stranded β sandwich. In B domain, the βC' and βC" strands are part of the interface in A/B5 contact. All three domains contain the αA helix. The first digit for numbering the amino acid residues is the chain identifier: 1 stands for the S subunit and 2 is for the L subunit. From Lin *et al*. (1999). (See Color Insert.)

TABLE II
SUBUNIT CONTACTS

Contacts (Subunit 1/Subunit 2)	Subunit 1	Subunit 2	
	# Residues	# Residues	Total buried surface area (Å^2)
A/B5	31	25	1573.4
C/B5	35	39	2207.3
A/C	29	31	1734.0
A/A5	51	45	2687.4
A/B	23	27	1362.4
B/C	33	26	1678.6
C/B2	31	35	1939.6
C/C2	42	42	2285.0

domains. Appropriately, the CPMV particle is best described as a pseudo $T = 3$ particle with the B and C domains clustered about the icosahedral threefold axes and 5A domains clustered at the pentamer axes. The domains are highly modular and all of the unique contacts between them are described in Fig. 1A and Table II. The extensive interactions within the pentamer, especially between A and A5 subunits, suggest that CPMV assembly may be similar to picornaviruses with the pentamer of large and small subunits as the assembly unit (Table II). Figure 6 illustrates the remnants of quasiequivalent contacts preserved at the A–B5 and C–C2 interfaces that are quasiequivalent in a true $T = 3$ surface lattice and the region close to the ABC junction, which is a quasi three fold axis in the $T = 3$ structure. The freedom offered by the separately evolving domains changes the pseudoequivalent interfaces dramatically, but the modular nature of the assembly units is largely maintained.

4. Structural Studies of Components

Not surprisingly, the pure M component produced crystals that were isomorphous with the MIX crystals and the structure was virtually indistinguishable from the MIX crystals.

The B_L component is the high-density form of CPMV in which endogenous polyamines were exchanged for Cs^+ ions (Virudachalam *et al.*, 1985). It also crystallized isomorphously with M and MIX. Comparing the B_L electron density with MIX and M revealed one significant difference. The electron density for a striking pentameric annular structure in M and MIX was virtually absent in the

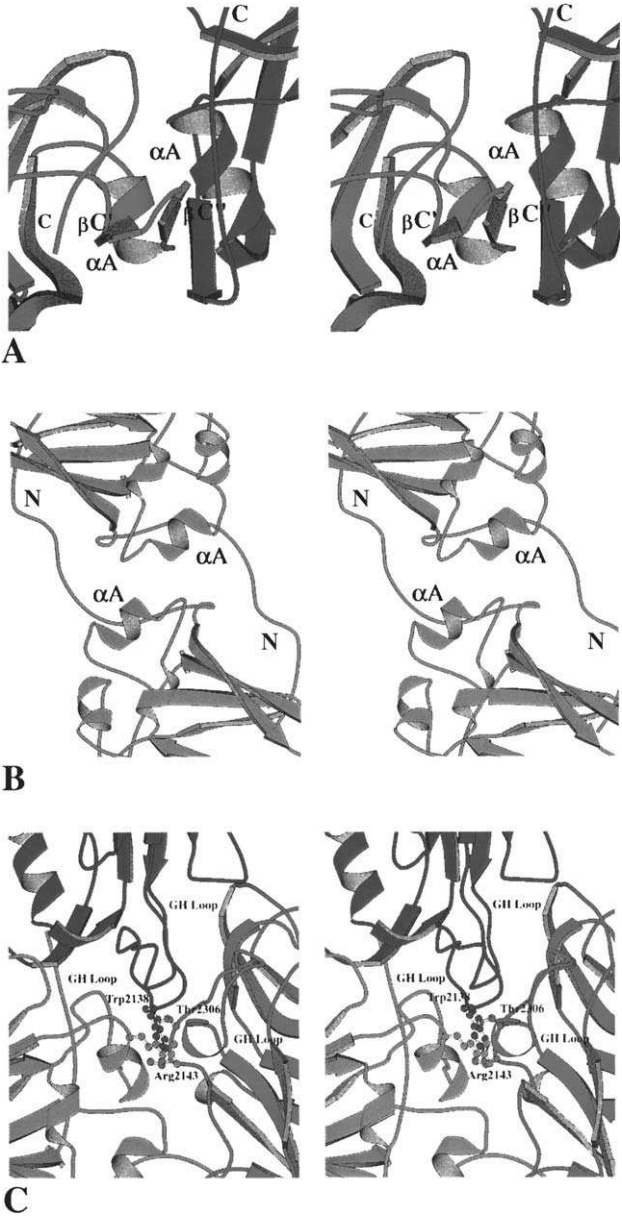


FIG 6. Stereo views of pseudoequivalent subunit contacts in the CPMV capsid. (A) The subunit contacts at the A/B5 interface. αA helices are packed side by side, and βC'βC" strands of the B domain, which are perpendicular to the long dimension of the β

B_L component (Fig. 7). In all other respects the structures were indistinguishable.

5. $P = 3$ vs $T = 3$

On the basis of virion size, protein composition, low-resolution X-ray crystallography, and electron microscopy, it was proposed that CPMV would have a capsid similar to $T = 3$ plant viruses except that it would have the three gene products in the icosahedral asymmetric unit: two being associated as a single polypeptide, the L subunit, and one constituting the S subunit (Schmidt *et al.*, 1983). The genome sequence and organization of CPMV suggested that it was also related to the picornaviruses (Franssen *et al.*, 1984; Lomonossoff and Shanks, 1983; van Wezenbeek *et al.*, 1983;). The 3.5-Å structure of CPMV (Stauffacher *et al.*, 1987) fulfilled this expectation.

The surface lattice of CPMV differs from true quasiequivalent $T = 3$ virus capsids observed and predicted previously by Caspar and Klug (1962). The asymmetric unit of a $T = 3$ particle is composed of three subunits of identical sequence occupying slightly different environments. The three β sandwich domains in CPMV occupy the same lattice positions as quasisymmetrical subunits in the asymmetric unit, but they are of a completely different chemical sequence. This variant of quasiequivalence, which is observed in picorna and picorna-like virus capsids, is termed pseudo $T = 3$ or $P = 3$ architecture (Rossmann and Johnson, 1989). The difference between $T = 3$ and $P = 3$ capsids results in the relaxed requirements for subunit interactions in a particle composed of three gene products, eliminating the need for the molecular switching observed in $T = 3$ viruses.

The quaternary structure of CPMV is best described as a combination of those observed in two different types of $T = 3$ viruses.

sandwich, interact tightly with the C terminus of the A domain. (B) The icosahedral twofold axis relates the αA helices at the C/C2 contact. The N terminus of the C domain extends past the axis and interacts with the twofold-related C domain, providing additional stability to the interaction. (C) At the pseudo threefold axis, all the interacting elements are from the GH loops of each of the three domains. However, there is no threefold symmetry relating the conformations in this $P = 3$ virus. The GH loop of the A domain (blue) is especially dissimilar to those of B (red) and C (green) domains, which correlates with the significant deviation from quasisymmetry of the A domain. Trp1138 of the A domain is interlocked by the hydrophobic region of Arg2143 of the C domain at the exterior and Val2305 and Thr2306 of the B domain at the interior. From Lin *et al.* (1999). (See Color Insert.)

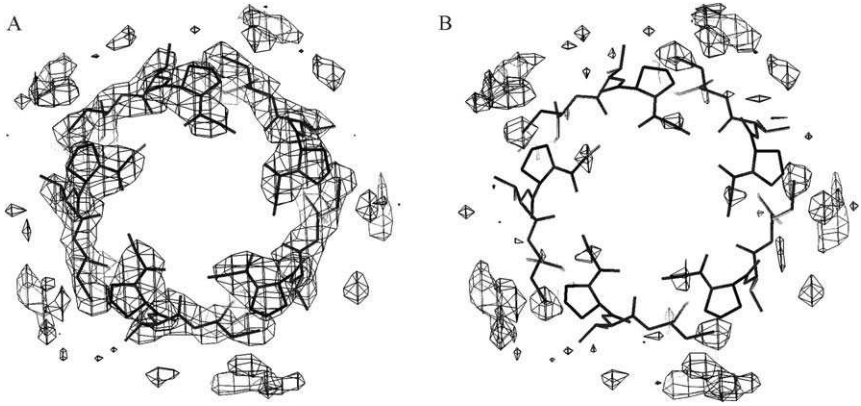


FIG 7. An interaction at the pentameric contact. Fivefold-related N termini of the S subunits form a pentameric annulus inside a channel-like structure along the fivefold axis of the viral capsid. This annulus is perturbed in the B_L component, which has Cs^+ bound to the RNA. (A) A section of the averaged electron density map of the CPMV MIX component looking down the fivefold axis from the capsid exterior. The electron density is defined, and the annular structure can be modeled with the N termini of the S subunit. (B) The model of annuli derived from the MIX component structure is superimposed with the electron density map of the B_L component. It is apparent that the electron density associated with annuli is ill defined in the B_L component, which is permeable to Cs^+ ions (Virudachalam *et al*., 1985). Both electron density maps are contoured at 1σ. From Lin *et al*. (1999).

Southern cowpea mosaic virus (SCPMV, previously known as southern bean mosaic virus) is a $T = 3$ virus and the three domains of its asymmetric unit are of an identical chemical sequence (Johnson *et al*., 1976; Silva and Rossmann, 1987). Obeying near perfect quasi threefold symmetry, the three domains in the icosahedral asymmetric unit are positioned with the long dimension of the β sandwich tangent to the spherical surface (Fig. 8A). This construction creates a near-spherical particle with the maximum internal volume for a given sized subunit. The internal volume of 7×10^6 Å^3 is adequate to package the monopartite SCPMV genome. In contrast, the β sandwiches of cowpea chlorotic mottle virus (CCMV), another $T = 3$ virus, stand nearly on end with the long dimensions of the β sandwiches roughly parallel to the adjacent fivefold or quasi sixfold symmetry axes (Fig. 8B), placing the long dimension of the subunit nearly radial to the spherical surface. This CCMV quaternary structure produces bold morphological features easily visible in an electron microscope (Speir *et al*., 1995), probably providing an advantage at some stage in the virus life cycle, but at the cost of packing capacity for its genome. The subunits of SCPMV

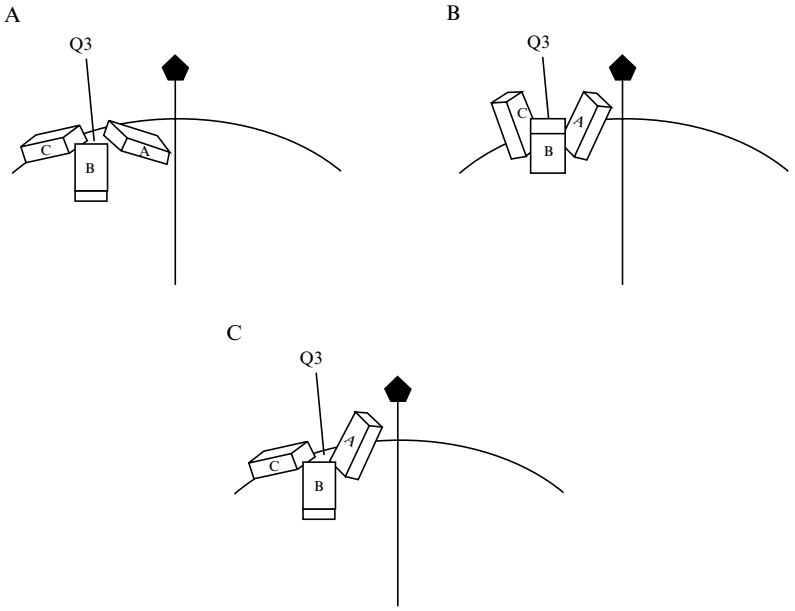


FIG 8. Schematic illustrations of quasi threefold-related subunits of virus capsids. The semicircles represent part of the spherical surfaces defined by the center of the subunits. Each brick represents a domain—A, B, or C—as labeled. The viral fivefold and quasi threefold axes are also shown. (A) A schematic representation of southern cowpea mosaic virus (SCPMV). The quasi threefold-related domains lie with their long dimensions along the sphere, producing a smooth surface and large internal volume for genome packaging. (B) A schematic representation of cowpea chlorotic mottle virus (CCMV). The subunits stand on end, producing bold protrusions on the viral surface. (C) A schematic representation of cowpea mosaic virus (CPMV). The different chemical compositions of three domains allow them to be arranged without the constraint of a quasi threefold axis. While the long dimensions of B and C domains are tangent to the sphere as in SCPMV, the A domain stands on end to form protrusions on the viral surface like CCMV. Consequently, the organization of CPMV capsid protein is a combination of that of SCPMV and CCMV. From Lin *et al*. (1999).

and CCMV are comparable in size, yet the internal volume of CCMV is smaller at about 5.2×10^6 Å^3. As a result of the different subunit arrangements, the packing capacity of SCPMV is approximately a third larger than that of CCMV. The relative difference in the internal volume of the two capsids correlates with the relative size of the packaged genomes. The genome of SCPMV is an RNA molecule of 4149 nucleotides (Sehgal, 1995). The CCMV genome is tripartite with two large RNA molecules packaged separately and one small genomic RNA packaged with a subgenomic RNA, all in isomorphous isometric

particles. The largest CCMV RNA packaged is 3234 nucleotides, roughly two-thirds of the SCPMV RNA, which is similar to the relative difference in the internal volume of the two viruses.

CPMV has two domains (B and C) oriented as in the SCPMV capsid and one domain (A) forming pentamers strikingly similar to CCMV (Fig. 8C). To date, such a combination of quaternary organizations is only observed in viral capsids with the three subunits formed by different polypeptide sequences. CPMV and SCPMV capsids are similar in size. However, CPMV RNA1, the larger RNA molecule comprising the CPMV genome, is 5889 nucleotides and about 40% larger than SCPMV RNA. Unlike SCPMV, the interior of the CPMV capsid is not a spherical enclosure. Deep pockets were found in the capsid facing the interior. This region of the protein shell bound approximately 660 nucleotides in BPMV (Chen *et al*., 1989; Lin *et al*., 2003) and apparently increased the packing capacity. The RNA-binding pocket could only form at the subunit interfaces at the interior between two different domains, B5 and C.

6. Comparison with Picornaviruses

CPMV and picornaviruses share similar tertiary and quaternary structures. However, their capsid protein sequences can only be aligned (Fig. 2A) with three-dimensional structural superposition. VP1 of picornavirus is equivalent to the S subunit of CPMV and occupies the A position in the icosahedral shell. Although VP2 and VP3 are two separate polypeptides and the L subunit is a single polypeptide, the L subunit β sandwich domains are analogous to VP2 and VP3 domains that occupy the C and B5 positions in the $P = 3$ icosahedral lattice (Figs. 1 and 4). Despite the overall similarity in capsid architecture, there are significant differences between the two viruses. These include the termini of each subunit, the interior of β sandwich domains of the VP1 and S subunit, the extra C'C" strands of S subunit, and the loops connecting the β strands. Such differences in structure reflect the differences in the infection of the animal and plant viruses.

The infection of picornaviruses involves receptor binding, endocytosis, and release of viral genome into the target cell (Rueckert, 1985). In contrast, CPMV infection is more straightforward and depends on an insect vector that delivers the virus particle directly into the plant cell, without the explicit processes associated with the entry into animal cells. The infection, therefore, does not require the delicate mechanism of interaction with the host cell, fine tuning of the stability, and conformation of the viral capsid. The simplified biology is reflected in the CPMV capsid structure.

The absence of receptor binding in CPMV is likely the explanation for the less corrugated surface of its capsid compared to picornaviruses. It is particularly obvious with the absence of CPMV residues that form the south wall of the receptor-binding site in picornaviruses. Consequently, there is no discernible receptor binding "canyon" as observed in some picornaviruses (Rossmann, 1989). Moreover, the location for binding the receptor is occupied by the additional βC' and βC" strands of the A domain, as if the receptor site is permanently occupied.

The so-called "pocket factors" are frequently found binding at a site inside the β sandwich domains of VP1 of picornaviruses, which can be displaced by antiviral drugs that stabilize the capsid and prevent infection (Smith and Baker, 1999; Smith *et al*., 1986). Binding of these drugs inhibited the uncoating of the RNA and made the capsid resistant to proteolysis (Lewis *et al*., 1998). In the CPMV S subunit, the analogous site is filled with hydrophobic residues, resembling an endogenous pocket factor that stabilizes the particle permanently. No antiviral drugs were found binding to CPMV.

CPMV subunits interact in a modular fashion with contacts mainly involving residues on the side of the β sandwich. In contrast, picornavirus subunits have additional extensive interactions with N and C termini, especially between VP1 and VP3 subunits, which possess extensions at both termini (Rossmann *et al*., 1985). Such interactions can occur only after proteolysis cleaves VP2 and VP3 into separate polypeptides. The C and B5 domains in CPMV are never cleaved, eliminating the free N-terminal end of B5, the analogue of VP3. Moreover, termini of the A domain and C terminus of B5 domain fold away from each other. Without extensive interactions of termini between subunits, there appears to be no major barrier for the CPMV capsid to dissociate and release the genome after entering the cell.

Detailed comparisons of picorna-like plant and animal virus capsids clearly demonstrate that the two capsids are related by evolution, while the sophistication of the capsids directly reflects the complexity of the respective virus life cycles. The CPMV particle can be viewed as a relatively inert and less elaborate delivery vehicle for the viral genome, whereas the picornavirus capsids are nano machines with extraordinary control elements built into their organization for sensing their environment and responding to it. The robust nature of CPMV, however, makes it refractory to many mutations and stable in nonaqueous solvents, leading to its use as a platform for chemistry and materials sciences, as discussed later. A potential use for this

icosahedral particle as a scaffold for visualizing attached exogenous proteins was first suggested by the combination of crystallography and EM described in the next section.

B. Structural Studies of CPMV/Antibody Complex by Electron Microscopy and X-Ray Crystallography

X-ray crystallography is not always suitable for the studies of large complexes of macromolecules to near atomic resolution as often only components are amenable for crystallization. Cryoelectron microscopy, however, is ideal for studies of large molecular assemblies, albeit only to intermediate resolution. It is a powerful approach to investigate the structures of complex macromolecules with the combination of electron microscopy for the analysis of large assemblies to intermediate resolution and X-ray crystallography of the constituents at near atomic resolution. The electron density from the image reconstruction defines the envelopes into which component structures to high resolution can be fitted. Consequently, the structures of a large complex can be studied at pseudo atomic resolution without crystals of the complex. This approach is now widely used for structural studies of macromolecular assemblies and was pioneered in the structural investigation of CPMV complexed with its cognate monoclonal antibodies (Porta *et al*., 1994b; Wang *et al*., 1992).

Bindings of two monoclonal antibodies (McAb), 5B2 and 10B7, which had high affinity to CPMV in DAS-ELISA experiments, were investigated (Porta *et al*., 1994b). Both F_{ab} fragments and intact IgG molecules were used to form complexes with the virus. Formation of the complex with McAb 5B2 was straightforward. The virus and the McAb at a ratio of 1:600 were incubated at 37 °C for 2 h and the unbound antibodies were separated from the complexes by centrifugation in sucrose gradients. The complex with McAb 10B7 was less stable, and a brief treatment with glutaraldehyde at a final concentration of 0.1% was employed to prevent the shedding of McAb from the complex. Reduction of IgG (1:60) was required to minimize the aggregation of virus in the formation of virus/IgG complexes.

Images of the virus/antibody complexes were generated with cryoelectron microscopy and image reconstruction, which revealed the overall morphologies of these structures (Fig. 9). The complex structures with the two F_{ab} fragments were nearly indistinguishable, yet the two IgG molecules were different in their behavior in solution and when digested with papain, eliminating any possibility that they were the same. The appearance of the IgG complex showed that the

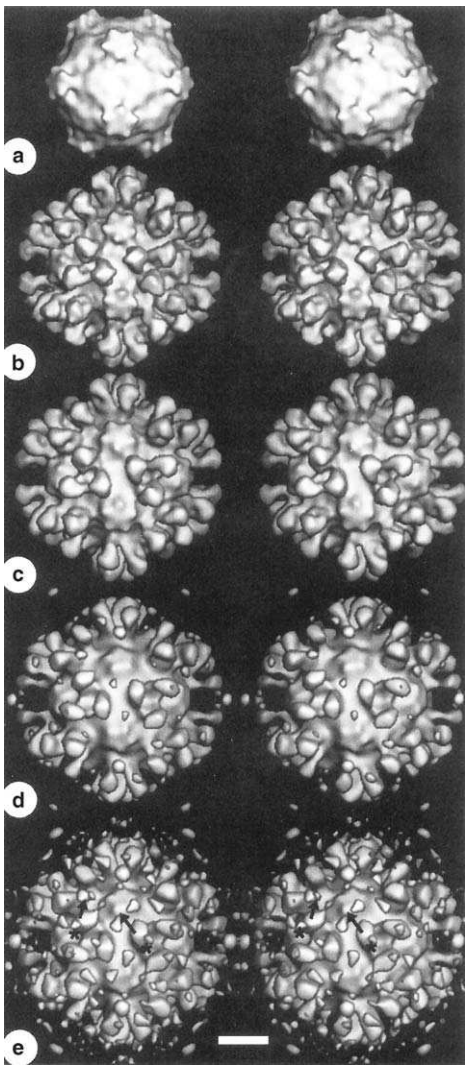


FIG 9. A gallery of stereo images of reconstructed densities of native CPMV and CPMV complexed with F_{ab} fragments or IgG. The reconstruction with F_{ab} fragments was at ~23 Å resolution, and the reconstruction with IgG was at ~30 Å resolution. (a) Native CPMV; (b) CPMV complexed with F_{ab} from McAb 10B7; (c) CPMV complexed with F_{ab} from McAb 5B2; and (d) CPMV complexed with IgG from McAb 5B2. This is contoured at a higher level than the reconstructions shown to emphasize the F_{ab} density; (e) same reconstruction as (d), but contoured at the same contour level as in (a), (b), and (c). The strong density features that appear as "islands" are probably preferred positions for unbound F_{ab}, and asterisks indicate the F_c position. Bar: 5 nm. From Porta *et al*. (1994b).

binding was monodendate. The F_c portion of the IgG and the F_{ab} fragment that was not attached to the viral surface had sufficient mobility such that they were detected only marginally in the reconstruction. In addition to the density of the bound F_{ab}, in the virus–IgG (5B2) reconstruction there were two regions of density that were clearly above background, but appeared as "islands" of density that were not visibly attached to the virus surface or the F_{ab} that was in contact with the virus surface. One of these regions suggested the approximate average position of the unbound F_{ab} of the IgG. The mobility of the F_c fragment made assignment of density for this portion of the IgG more speculative. A molecular model of F_{ab} (Kol) (Marquart *et al.*, 1980) and the refined structure of CPMV were fit into the electron density of the image reconstruction of CPMV/F_{ab} 10B7 (Fig. 10). Such modeling allowed the footprint of the F_{ab} to be defined accurately on the CPMV surface and thereby identified residues that were likely to be involved in the interaction with the antibody (Fig. 11). Lys234 (C domain) and Thr2207 (B domain) were particularly prominent within this F_{ab} footprint. These residues were located on the outermost loops of the B and C domains, respectively. The footprints of the F_{ab} and IgG of 5B2 and F_{ab} of 10B7 were nearly identical, indicating that there was no difference in binding of the IgG and F_{ab} of 5B2 and also that 10B7 bound to virtually the same site as 5B2.

Although the F_{ab} (Kol) model fit the density quite well, it was impossible a priori to determine unambiguously its orientation in terms of the respective positions of the heavy and light immunoglobulin chains. Either of two orientations about the pseudo dyad axis that related light and heavy chains fit the density equally well. The F_{ab} extended radially from the surface of the virus about 70 Å, and the shape of the density indicated that the elbow angle was nearly linear (Fig. 10A). The footprint (Fig. 11) had an ellipsoidal boundary with major and minor axes of 40 and 20 Å. The approximate center of the footprint was located 25 Å from the icosahedral threefold axes. Roughly 30 residues were entirely under the footprint of the F_{ab} and 6 others were partially covered (Table III). Because these residues were located on two different L subunits, the binding site spanned a subunit interface (Fig. 11C). Thus, the capsid quaternary structure was required for the binding of the antibodies, which was consistent with the fact that 5B2 and 10B7 reacted only with sandwich type ELISAs. Lys234 (C domain) and Thr2207 (B domain), the most prominent residues within the F_{ab} footprint on CPMV, were spatially equivalent to the corresponding amino acids that defined the 3B antigenic site on poliovirus (Mosser *et al.*, 1989; Page *et al.*, 1988). If either of these two residues was changed

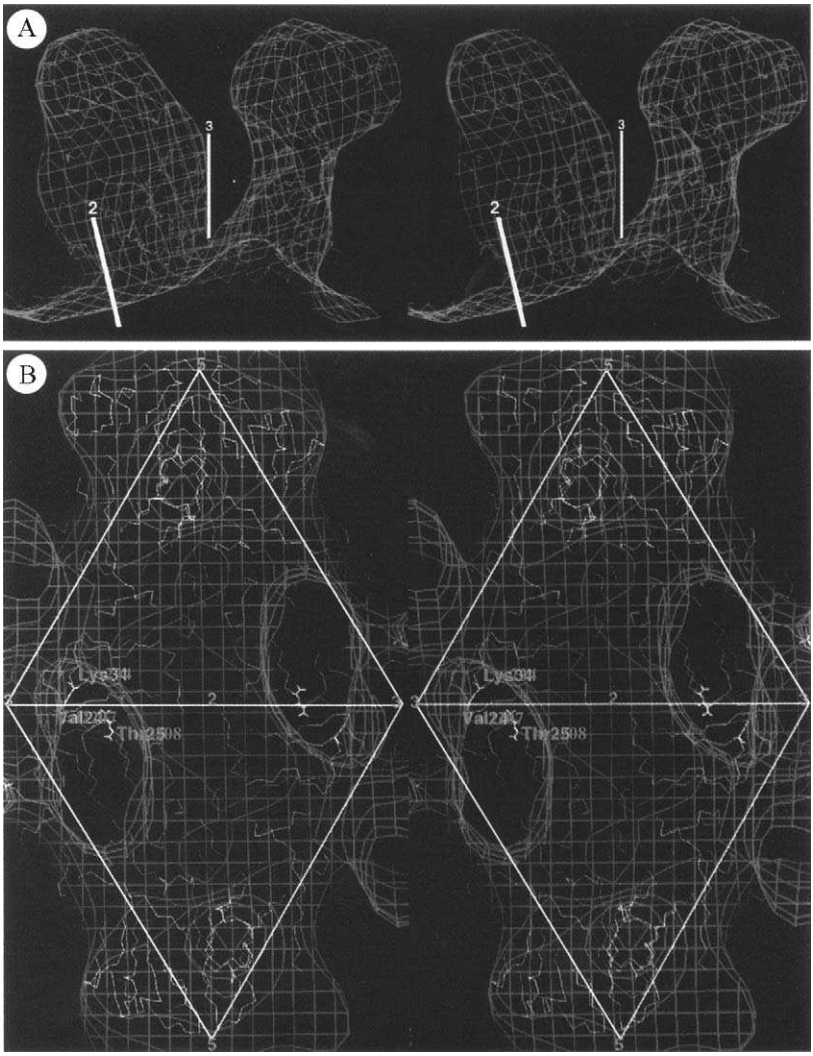


FIG 10. (A) Stereo view of density derived from the reconstruction of CPMV complexed with F_{ab} 10B7 shown in Fig. 9 compared with the atomic model of F_{ab} Kol used as a generic model for the F_{ab}. (B) Stereo view of a detailed footprint of the F_{ab} on the surface of CPMV showing the residues in contact with F_{ab} 10B7. Lys234, Val2207, and Thr2208 are highlighted in the Cα backbone. The electron density of the cryo-EM reconstruction is shown as blue contours, the VP2 domain is shown in green, the VP3 domain is shown in red, and VP1 (small subunit) is shown in yellow. From Porta *et al*. (1994b). (See Color Insert.)

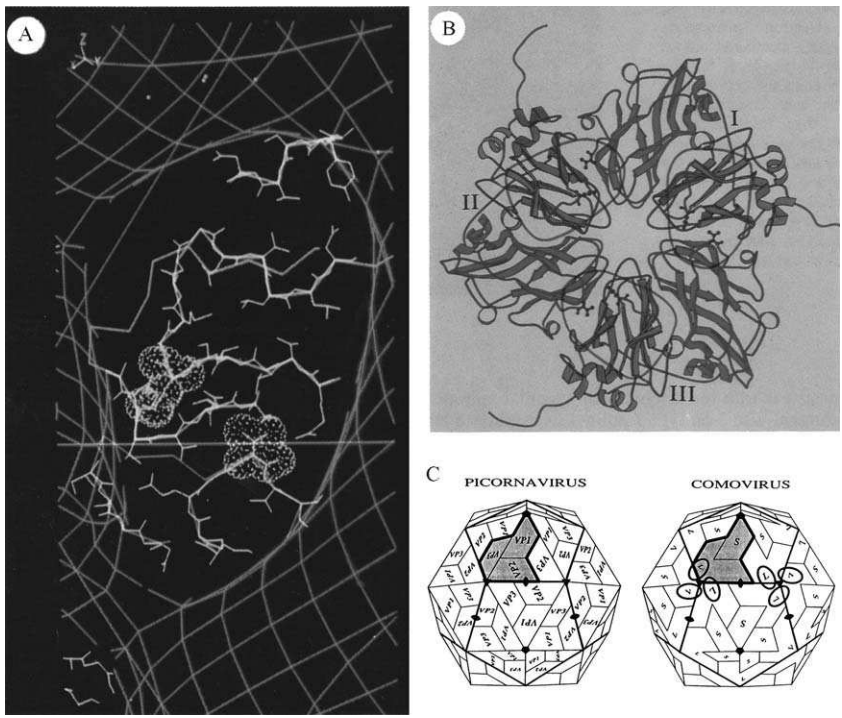


FIG 11. (A) The F_{ab} footprint on the virion surface showing regions of the CPMV peptide chain that lie under the F_{ab}. The region in red corresponds to the B domain of the threefold-related L subunit. Main chain and side chains of the residues that may interact with the F_{ab} are shown in yellow. Amino acids displaying surface rendering (Lys234 and Thr2207) are spatially equivalent residues 72 and 76 in the poliovirus VP2 and VP3 domains. Mutations of either of these residues in poliovirus prevented neutralization by poliovirus monoclonal antibodies (Page *et al*., 1988). (B) Ribbon diagram of a trimer of the L subunit. Ellipsoids identify the viral surface in contact with F_{ab}. Side chains shown correspond to residues with surface rendering in A. The ellipsoid on the right, covering I and III, is in the same orientation as in the ellipsoid in A. (C) A comparison of picorna and comovirus capsids with 6 (of the 60) F_{ab} footprints on the comovirus capsid represented by ellipsoids. The picornavirus particle is formed from 60 copies each of three different, but similar, gene products labeled VP1, VP2, and VP3. The comovirus capsid is formed of 60 copies each of an L and an S subunit. L subunits form two β sandwichs corresponding to VP2 at the amino end and VP3 at the carboxyl end, whereas S corresponds to VP1. The footprint spans a subunit interface with the smaller part of it interacting with the VP2 portion of one L subunit and the larger part of it interacting with the VP3 portion of a different L subunit. Adapted from Wang *et al*. (1992). (See Color Insert.)

TABLE III
RESIDUES IN CONTACT WITH FAB FRAGMENTS 5B2 AND 10B7

VP2 domain	VP3 domain	VP3 domain (cont.)
Leu232	Lys2200	Arg2212
Ser233	Leu2201	Arg2213
Lys234[a]	Thr2202	Met2214
Ala235	Phe2203	Ala2224
Met236	Pro2204	Thr2225
Gly238	Gln2205	Thr2336
Gly239	Gly2206	Gly2337
Ser2154	Val2207[a]	Gly2341
Gly2155	Thr2208[a]	Asp2342
Pro2160	Ser2209	Leu2369
Thr2161	Glu2210	Leu2370
Thr2162	Val2211	
Asp2163		

[a] These residues (highlighted in Figure 11) are spatially equivalent to the residues that define site 3B of Poliovirus as determined by escape mutation analysis (Page *et al*., 1988).

to a different amino acid in poliovirus, the virus was no longer neutralized by a particular group of McAbs. Analysis of the 3B antigenic site in poliovirus showed that it was dependent on the assembly of multiple 14S subunits made up of a pentamer of the capsid polypeptides (Rombaut *et al*., 1990). This result was consistent with the nature of the site determined in CPMV. Human rhinovirus 14 (HRV14) and poliovirus shared a number of geometrically equivalent antigenic sites as determined by escape mutations. However, neutralizing immunogenic site III (NIm-III), the site on HRV14 that corresponded most closely to the 3B site of poliovirus, was confined to a single subunit, VP3 (Mosser *et al*., 1989). The C domain of the CPMV large subunit constituted roughly two-thirds of the footprint for the 5B2 and 10B7 antibodies, thus this binding site was probably similar for HRV14 as well as for poliovirus.

The NIm-1A site on HRV14 was analyzed by Smith *et al*. (1993b) with F_{ab} derived from McAb 17 using the same procedure for 5B2 and 10B7 binding to CPMV. This site differed in two ways from the 3B site of poliovirus. The F_{ab} 17 binding site was on subunit VP1 and all the regions of interaction between this F_{ab} and the virus were confined to a single subunit. In a subsequent report, Smith *et al*. (1993a)

showed that McAb17 IgG bound in a bidentate fashion that maintained the icosahedral twofold symmetry. The distance between the centers of gravity of the twofold-related NIm-1A footprints was 110 Å. This was much longer than the corresponding distance between the twofold-related footprints in CPMV, which was 78 Å. Although the 5B2 site on CPMV was situated adjacent to twofold symmetry axes, there was no density connecting the twofold-related F_{ab} units. Each binding site must therefore correspond to an IgG with one F_{ab} attached and the other unattached. Mosser *et al*. (1989) considered the likelihood of bidentate binding for all the monoclonal antibodies that they analyzed for poliovirus and rhinovirus. They concluded that monoclonal antibodies binding to site 3B could not form a twofold axis-related bidentate interaction with the virus because the distance between these binding sites was less than 90 Å, the minimal distance estimated for bridging by an IgG (e.g., Icenogle *et al*., 1983). The monodentate binding observed with the IgG of 5B2 supported this conclusion.

The antigenic surface of another comovirus, BPMV, was also investigated but with the synthetic peptide approach (Joisson *et al*., 1993). This analysis revealed a site corresponding to NIm-1A in rhinoviruses (see earlier discussion), but was sensitive only to the portion of the 5B2 site on CPMV that was on the C domain (the peptide generating reactive antibodies contained residues 231–242 of the BPMV C domain).

Knowledge of the antigenic sites on the surface of plant viruses has significance at two levels. First, epitopes may be classified as "natural" or "acquired" by comparing their character and location on related plant and animal viruses. Because plant viruses are not under surveillance of a circulating immune system, the site common to 5B2 and 10B7 on comoviruses and 3B on poliovirus may result from a quaternary structure that is shared by all viruses with a picorna-type capsid. In contrast, the NIm-1A site on rhinovirus appears to have evolved specifically for the purpose of "decoying" the immune system. This site and others consist of surface loops that are readily mutable without affecting other functions of the capsid structure. A second level of importance relates to the use of plant viruses as "expression systems" for animal virus epitopes through the genetic engineering of chimeric plant viruses that express regions of polypeptide chains known to be antigenic on the surface of the animal virus (see next section). The knowledge of naturally occurring antigenic sites on the surface of plant viruses may assist in making the most rational constructs or altering acquired immunity that may result from previous exposure of an animal to a natural version of the virus. In CPMV, for instance,

changing Lys234 or Thr2207 in the 5B2 footprint may reduce natural immunity to native or altered CPMV particles.

The structural studies of the CPMV/F_{ab} complex illustrate the power of combining the moderate resolution of cryo-EM reconstructions of a complex with the high-resolution structures of the component determined by X-ray crystallography. Although the actual resolution of the reconstruction was ~23 Å, the effective resolution with the combined information was much higher because the atomic resolution structures of the components were known and could be docked accurately within the EM density. A shift of the F_{ab} fragment by as little as 5 Å significantly reduced the quality of the fit of the atomic model to the cryo-EM density (Liu *et al*., 1994). The footprint of the F_{ab} on the viral surface was defined with even greater precision than the overall fit of the F_{ab} model to the density, leaving virtually no doubt regarding which residues of the virus were covered by the F_{ab}. The combination of cryo-EM and crystallographic methods is analogous to the refinement of protein structures. Structural data for typical proteins are available to only 2.5 Å but it is possible to refine these structures to within a fraction of an angstrom because the detailed structures of amino acids and small peptides are known, which dramatically constrains the stereochemistry of the protein. Similarly, the known high-resolution structures of the components (the F_{ab} fragment and the virus) dramatically constrain the interpretation of cryo-EM reconstructions.

C. Chimeric CPMV

Synthetic polypeptides composed of about 10–40 amino acids have a broad spectrum of use, ranging from antigens for epitope-based vaccines to specific inhibitors of cell surface interactions. While short peptides are often immunogenic, their efficacy can be improved by presenting them as multiple copies on the surface of large carrier molecules (Francis, 1990, 1991). Genetic insertion of an appropriate sequence into a protein-encoding gene and then expressing the altered gene in a heterologous expression system can achieve similar results with the protein carrying the polypeptide (Lomonossoff and Johnson, 1995). Seminal work of this type was done with the hepatitis B core protein, which assembled spontaneously into an icosahedral particle when it was expressed in *Escherichia coli* (Clarke *et al*., 1990). Because the structure of this protein was unknown, the genetic constructs were made by adding the appropriate oligonucleotides to the 5′ and 3′ ends of the hepatitis B core protein gene. The resultant

polypeptides added to the N and C termini, fortuitously, did not interfere with assembly and were displayed as linear peptides on the virus surface with a biological efficacy exceeding that of the peptides attached chemically to other protein carriers. The first structure-based inserts of this type were made with poliovirus where the availability of the 2.8-Å X-ray structure (Hogle *et al*., 1985) suggested that inserts made in an exposed loop near the particle fivefold axes (βB-βC loop of VP1, see later) would be accessible on the surface of the particle. In contrast to the hepatitis B core protein system, an infectious clone of the virus (Racaniello and Baltimore, 1981) was used, and the genetically inserted polypeptide was amplified with the virus as it multiplied in the host (Burke *et al*., 1988). Although some of the inserts reduced or inhibited virus replication, many of the constructs multiplied at nearly normal levels, providing candidates for live-virus, peptide-based vaccines (Crabbe *et al*., 1990).

1. Chimeric CPMV Technology

CPMV capsids are particularly suitable for polypeptide presentation as they are stable and can be propagated in large quantities and purified with a straightforward protocol. The exploitation of this virus system for the expression of heterologous peptides requires the generation of infectious clones that can be altered in their coding sequences and subsequently transfected in plants to obtain the desired mutant viruses. The first infectious clones were generated by placing the promoters of T7 or SP6 RNA polymerase promoters in clones of the CPMV cDNA. Transcripts from *in vitro* experiments were used to transfect the plants for the production of mutant viruses (Eggen *et al*., 1989; Holness *et al*., 1989; Rohl *et al*., 1993; Vos *et al*., 1988). Significant improvement was made by the introduction of the 35S promoter from the cauliflower mosaic virus, which generated infectious cDNA clones and facilitated the production of CPMV mutants greatly (Dessens and Lomonossoff, 1993).

The βB-βC loop of the S subunit is highly exposed on the capsid surface, and a comparison of the sequence and conformation of this loop with two other comoviruses, BPMV and RCMV, showed a high degree of variability (Lomonossoff and Johnson, 1995). This suggested that this loop could be exploited for the insertion of different amino acid sequences without compromising normal virus function. Structural superposition of CPMV and human rhinovirus 14 showed that there were about 40 extra residues in the βE-βF loop of the VP2 subunit of HRV14 in comparison with the C domain of CPMV (Brennan *et al*., 1999), which indicated that this loop might also be amenable for the

insertion of exogenous peptides. Two other surface locations, βC'-βC" loops and the C terminus of the small subunit, were also determined to be suitable for the insertion of exogenous peptides (Taylor *et al.*, 1999b, 2000). Locations for the insertional mutagenesis are shown in Fig. 12.

Several guidelines are determined for the construction of viable and genetically stable CPMV chimeras (Porta *et al.*, 1994a; Usha *et al.*, 1993). First, the foreign sequence should be inserted as additions to the wild-type CPMV sequence and not used as replacements for the native residues. Second, methods for introducing foreign sequences that result in sequence duplications on either side of the insert are unsuitable, as loss of the insert occurred on passage due to homologous recombination. Third, the precise site of insertion within the sequences, notably in the βB-βC of the S subunit, can be important in maximizing the growth of the chimeras.

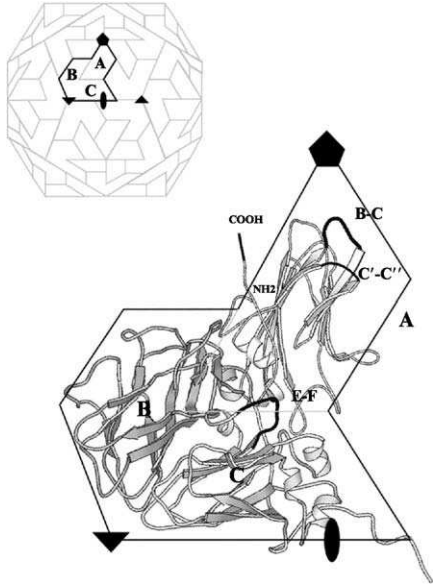


FIG 12. A ribbon drawing of the subunit forming the icosahedral asymmetric unit of CPMV and a diagrammatic representation of the virus particle in the upper left. Four primary sites are identified for insertional mutagenesis, which are bold loops (βB-βC, βC'-βC", and βE-βF loops), as well as the bold C terminus. Three of the sites are in the A subunit, the other, the βE-βF loop, is in the C domain. Adapted from Chatterji *et al.* (2002).

Large numbers of CPMV chimeras have been produced through the years. In most cases, the presence of heterologous sequences did not affect the ability of the viruses to grow in plants, and the yields of modified viruses were similar to those obtained from plants infected with the wild-type virus. The infection could be sap transmitted to healthy plants to allow efficient propagation of chimeric viruses. The genetic stability of several chimeras was determined, and inserts could be maintained intact through more than 10 serial passages, provided the size of the inserts was no more than 40 amino acids. Chimeric particles were shown to be stable at pH 1.0 and to survive incubation with concentrations of pepsin that completely degraded hemoglobin (Xu *et al*., 1996), which suggested that CPMV-based chimeras could retain their structural integrity during passage through the stomach, raising the possibility that they could form the basis of edible vaccines. Figure 13 illustrates diagrammatically the cloning, expression, and purification strategy for chimeric CPMV technology, as it is commonly called.

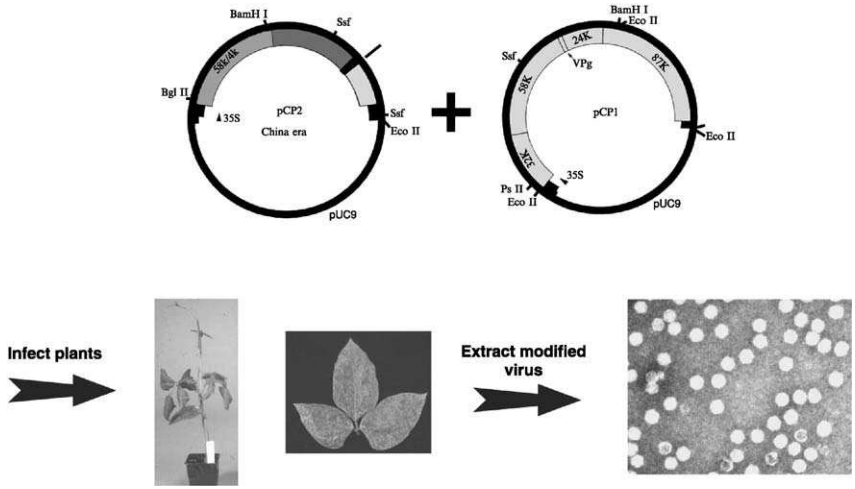


FIG 13. Scheme for the production of CPMV-based chimera. Two cDNA infectious clones, each encoding one of the viral RNA molecules, are under the control of the 35S promoter from cauliflower mosaic virus. Mechanical inoculation of both cDNA onto cowpea plants sets off the viral infection for the production of CPMV. Gram quantities of CPMV can be isolated in the laboratory setting. Adapted from Johnson *et al*. (1997).

2. Structure-Based Design for Better Presentation of Exogenous Peptides

One of the major advantages of CPMV as an epitope presentation system is the crystallization of chimeric particles and their structural studies by X-ray crystallography (Johnson *et al*., 1997; Lin *et al*., 1996; Porta *et al*., 1996; Taylor *et al*., 2000). Thus the CPMV provides a system for analyzing the effect of a positional presentation of a peptide on its immunogenicity. The sequence KDATGIDNHREAKL, which corresponds to the NIm-IA antigenic site of HRV14 (Sherry *et al*., 1986), is located in the βB-βC loop on the VP1 subunit and was chosen for the initial study. Because the structure of HRV14 was determined to 2.8 Å resolution (Rossmann *et al*., 1985), the conformation of this continuous epitope was known with high precision and could be compared to that on the CPMV surface and correlated to the immunogenicity.

The NIm-1A epitope was inserted at three locations in the βB-βC and βC'-βC" loops of the S subunit for structural and immunological studies. Insertion between Ala122 and Pro123 of the βB-βC loop of the S subunit produced the chimera CPMV/HRV II epitope (Porta *et al*., 1994a); insertion in between Pro121 and Ala122 of the same loop produced CPMV/L1 (Taylor *et al*., 2000), and CPMV/HRV-44-45$_1$ had the insertion in between residues 144 and 145 of the βC'-βC" loop of the S subunit (Taylor *et al*., 2000). Figure 14 shows schematically the generation of the CPMV/HRV II chimera.

Analysis by SDS–PAGE of preparations of CPMV/HRV II and CPMV/HRV-44-45$_1$ chimeras revealed that a high proportion of the S protein subunits were cleaved between the carboxy-terminal two residues of the inserted peptide. This proved to be a generic behavior of chimeras (Lin *et al*., 1996; McLain *et al*., 1995; Taylor *et al*., 2000), and the site of cleavage was independent of both the size and the sequence of the insert (Taylor *et al*., 1999a). The C-terminal portion of the S protein (termed S′) consisted of the last residue of the inserted epitope and the carboxy-terminal 192 residues of the CPMV S subunit, whereas the amino-terminal portion contained all but one amino acid from the inserted epitope linked to the N-terminal 22 amino acids of the S subunit. The two portions of the S subunit did not dissociate but were held together by noncovalent forces in the assembled particle (Lin *et al*., 1996). A consequence of this cleavage was that the inserted peptide was presented not as a loop, but with its C terminus free and probably effectively as an extended peptide.

CPMV/L1 was an exception. Despite a seemingly minor change of positioning, the insert from in between Ala122 and Pro123 of the

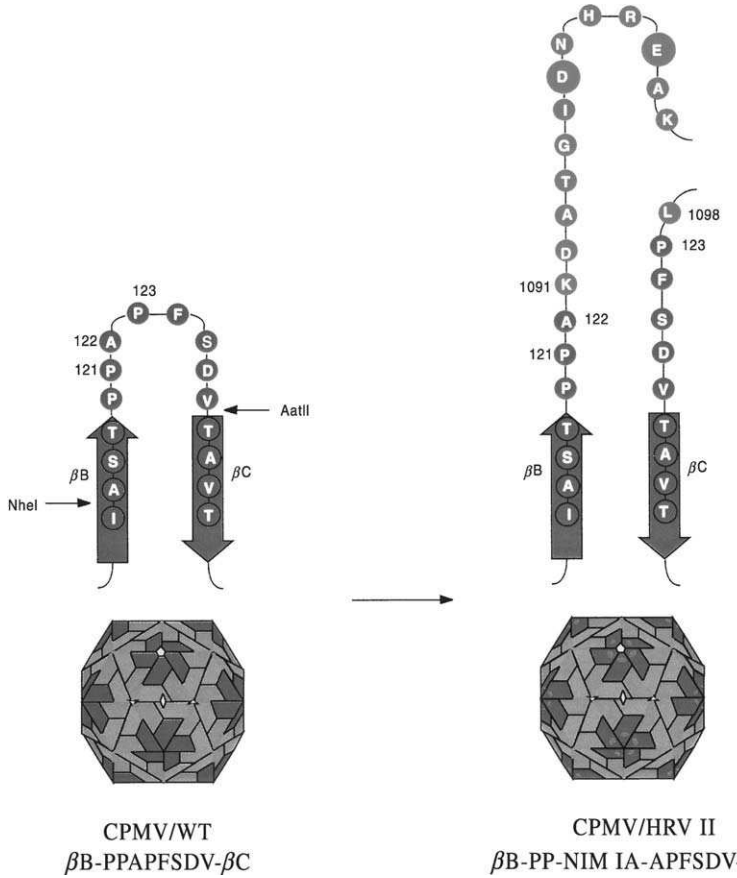


FIG 14. Generation of the CPMV chimera. (Top) Residues in the βB-βC loops of CPMV and the location of restriction sites, as well as residues in the chimera and the position of the spontaneous cleavage in the chimera. (Bottom) The location of the exposed loop of the chimera on the particle surface with the insertion represented in red. There are two unique restriction enzyme sites, *Nhe*I (natural) and *Aat*II (prepared by site-directed mutagenesis), in the region encoding the βB-βC loop of the small subunit in the infectious clone. These two sites are used in the cassette mutagenesis. Oligonucleotides are introduced to restore the native sequence of those residues and to insert the NIm-IA antigenic sequence of HRV14 between Ala122 and Pro123. Two residues, D1091 and E1095 (numbering from HRV14), which were important for the NIm-IA immunogenicity, are drawn as larger circles. The chimera thus generated has the foreign sequence presented at the pentamers of the virus capsid (lower right). The majority of the virus isolated is fragmented between K1097 and L1098. From Lin *et al*. (1996). (See Color Insert.)

S protein in CPMV/HRV II to between Pro121 and Ala122, SDS–PAGE analysis indicated that only a small percentage (<5%) of the chimeras were cleaved at the insert and the chimera displayed properties different from the cleaved insert (Taylor *et al*., 2000). This suggested that NIm-1A was presented on the CPMV surface as a closed loop, like the native presentation. This offered a novel opportunity for comparison with the open loop presentation and the immunogenicity of the two presentations.

Large crystals of CPMV/HRV II chimera were grown under conditions nearly identical to those used to crystallize the wild-type CPMV, and X-ray diffraction patterns with measurable reflections beyond 1.9 Å were observed (Lin *et al*., 1996). The native and chimera crystals were isomorphous, with 123 space group symmetry and $\mathbf{a} = 317.0$ Å. The particle position and orientation were defined by the crystal lattice with only the particle fivefold axis being not part of the crystal lattice. Interparticle contacts occurred along coincident threefold lattice and particles axes. The fivefold particle axes were fully exposed to solvent with adequate interparticle space in the crystal to accommodate the genetically inserted polypeptide, consistent with the formation of isomorphous crystals for the native and chimeric viruses. Conformation of the inserted loop did not appear to be affected by crystal contacts.

An electron density calculated as a difference map was computed. This map showed the difference in the electron density when the structural information of the chimera was subtracted from that of the wild-type CPMV. Positive density was seen for residues in the native structure that changed position due to the inserted polypeptide and negative density for regions occupied by residues in the chimera that were not occupied in the native structure. All significant positive and negative density in this map was found in the immediate vicinity of the βB-βC loop of the S subunit where the foreign sequence was inserted (Fig. 15). This demonstrated that the insertion created only local changes in the capsid structure.

For technical reasons, the negative density did not map the positions of all the inserted residues, and it was necessary to improve the phases with real space electron density averaging to obtain the complete density for the inserted portion of the structure (Fig. 16).

On the surface of HRV14, the structure of the NIm-1A epitope was dominated by intraloop hydrogen bonds, which were responsible for it adopting a convoluted structure. These hydrogen bonds were formed as a result of the NIm-1A sequence being presented as a loop with the first and last amino acids being only 9 Å apart.

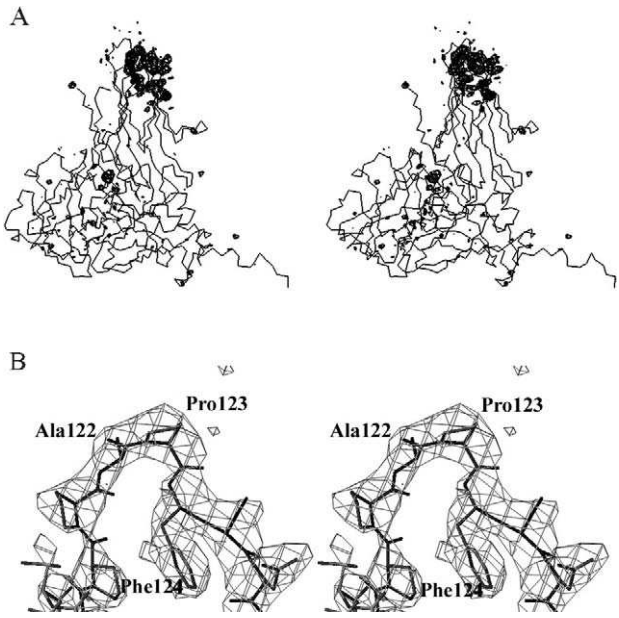


FIG 15. An averaged, difference electron density map with coefficients $(|F_{native}| - |F_{chimera}|)e^{i\alpha(native)}$ employed in the Fourier series. (A) A stereo view of the electron density for the entire icosahedral asymmetric unit showing that the only significant differences between native and chimera structures were at the βB-βC loop (top of figure). (B) A close view of the strong density observed for residues in the native structure (shown as the refined model) that changed position due to the inserted polypeptide. In the native structure, Phe124 was in a hydrophobic environment between residues in the loop connecting βB and βC strands and the loop connecting βH and βI strands. From Lin *et al*. (1996).

In contrast, the structure of the NIm-1A epitope on the surface of CPMV/HRV II was much smoother (Lin *et al*., 1996). This was a consequence of the cleavage that led to the loop adopting a less constrained configuration. In contrast to its native disposition in HRV14, residues at the beginning and end of the epitope were 19 Å apart (Fig. 17). The inserted sequence was very mobile, displaying high temperature factors and requiring low contour levels in the electron density map for full visibility.

Not surprisingly, CPMV/HRV-44-45$_1$ adopted a smooth conformation as in the CPMV/HRV II, albeit with different dispositions, as the insert was also cleaved. Although the electron density corresponding to the insert was discontinuous at 4.0 Å, probably due to the flexibility of the sequence, continuous density was obtained by averaging at 6.0 Å

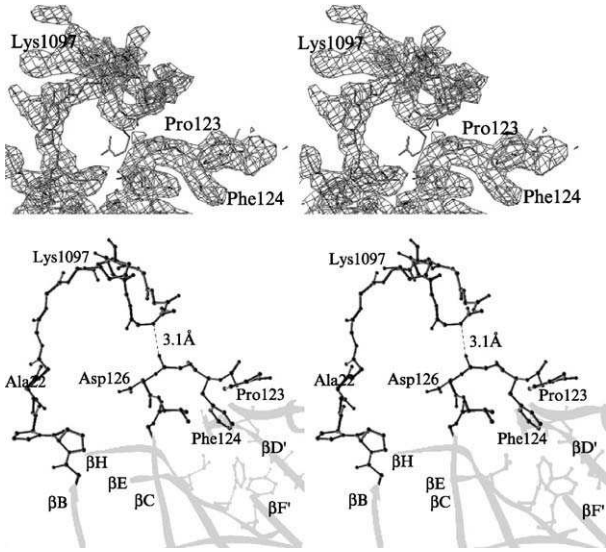


FIG 16. Electron density for the chimera particle. The main chain of the modified βB-βC loop was clearly defined in the averaged electron density map computed with amplitudes $F_{chimera}e^{i\alpha(\text{real space averaging})}$ and side chains of 12 of the 14 inserted residues were modeled unambiguously. The model is shown below and fit to the density above. β strands and side chains that comprise the immediate environment of Phe124 are also shown in gray. From Lin *et al*. (1996).

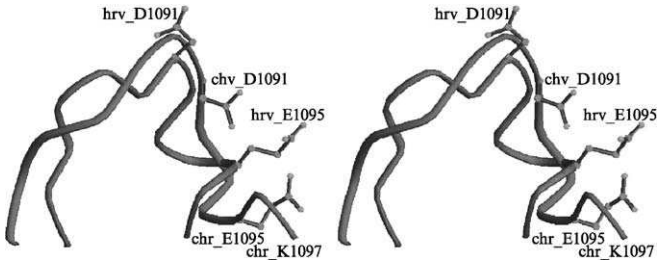


FIG 17. A stereo view comparing the chimera loop (in red) with the native NIm-IA loop of HRV14 (in gray); D1091 and E1095, which defined the NIm-IA epitope in that changes to either of these residues stopped the virus from being neutralized by specific monoclonal antibodies, are labeled in both loops. The NIm-IA loop displayed three turns, with the two ends of the loop 9 Å apart; the chimera loop was relatively smooth and 19 Å separate the two ends. From Lin *et al*. (1996). (See Color Insert.)

(Fig. 18), and a Cα backbone model corresponding to the inserted sequence was built into this density. Employing low-resolution data allows the definition of the statistical envelope occupied by the insert in the various particles of the crystal. At least two dominant conformations of the insert were identified: one with an extended conformation (Fig. 18A and 19) and the other folded as a loop with noncovalent interactions with the particle surface (Fig. 19). The extended insert folded away from the five-fold axis with its new C terminus interacting with Asn252 of the L subunit (Fig. 18A). The other conformation also involved interaction of the new C terminus, but it was with the new N terminus and thus formed a loop as if the cleavage did not occur. Both conformations were different from that adopted by the same sequence presented in the βB-βC loop of CPMV (Lin *et al.*, 1996) (Fig. 19), and the distance between the two ends of the extended conformation was significantly longer at 37 Å. In comparison, the same distance in CPMV/HRV II was 19 Å and it was 9 Å in the native environment. It demonstrated that the site of insertion in the virus particle could influence the structure of an insert. The conformations of the inserts in CPMV/HRV-44/45$_1$ were also distinctly different from the more compact structure adopted by the peptide on the surface of HRV14 (Fig. 19).

Noncleaved CPMV/HRV-L1 crystals diffracted X-ray to 3.3 Å resolution. The electron density around the insertion at 3.3 Å was discontinuous compared to that at 6 Å due to the mobility of the inserted epitope. Data were analyzed at 6 Å where a broad, but well-defined, statistical envelope of occupancy was defined. Figure 18B shows the electron density interpreted as the inserted peptide. As expected from biochemical data, the density was continuous. The epitope was presented as a closed loop (Fig. 19), as it was in HRV14, in contrast to the extended peptide found in CPMV/HRV II and CPMV/HRV-44/45$_1$. The mode of presentation, however, did not impose the native structure of the NIm-1A sequence as it could not be fitted into the electron density of CPMV/HRV-L1. The distance between the two ends of the insert was 23 Å, much longer than the 9 Å in CPMV/HRV II. Although the two ends were restrained due to the absence of cleavage, the intraloop hydrogen bonds did not form as in the native structure and the loop was fully extended.

Antisera for the three chimeras were raised against purified virions in rabbits to investigate the influence of the different modes of presentation on their immunogenic properties. To measure the level of anti-HRV14 antibodies in sera, their ability to bind to native HRV14 was tested by ACP-ELISA (Fig. 20). Results showed that antibodies raised

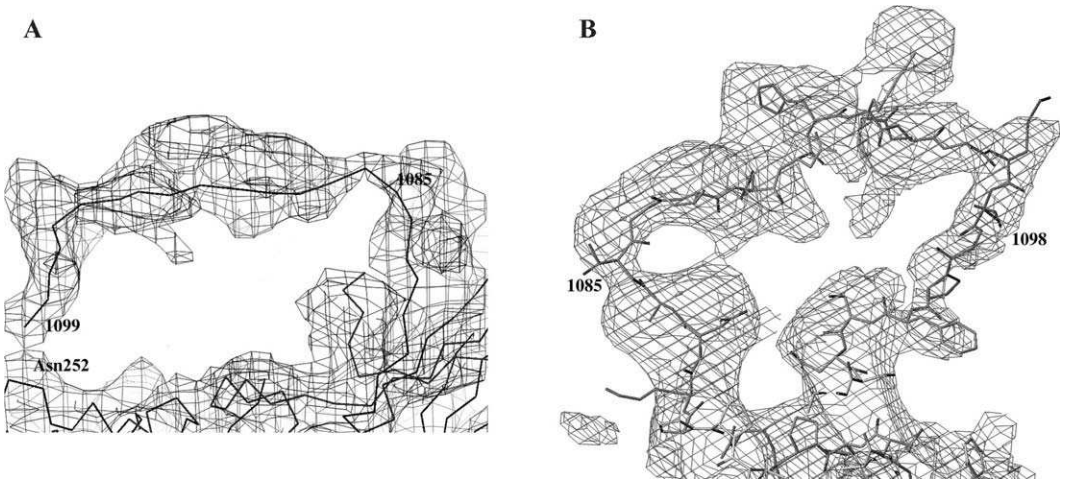


FIG 18. Electron density maps of CPMV/HRV chimeras. (A) CPMV/HRV-44/45$_1$. Electron density that was modeled for the extended conformation of insertion. The electron density is in chicken wire, and the model is made with Cα tracing in black. The length of the density can accommodate all the inserted residues, plus a residue (1099) at the end of the insertions, agreed with the biochemical analysis. A break of the density was obvious at the position where the cleavage occurred, and the new C terminus interacted with Asn252 of the large subunit. The electron density is contoured at 1σ. (B) CPMV/HRV-L1. Electron density that was modeled for the inserted loop. The density in chicken wire is continuous, and all the residues (1085 to 1098) of the inserted peptide can be fitted (in black). The density is contoured at 2σ. From Taylor *et al*. (2000).

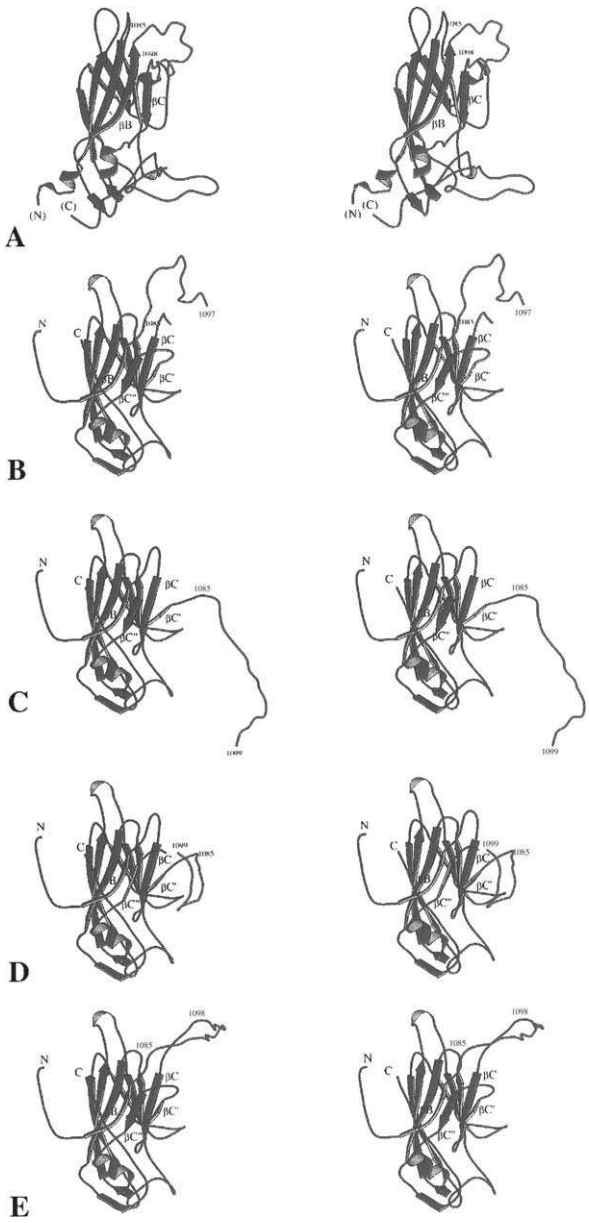


FIG 19. Stereo views of NIm-1A sequences presented on viral surfaces. NIm-1A sequences are in red. (A) VP1 of HRV14. The sequence in its native environment. N and C termini of VP1 are truncated in this presentation. (B) CPMV/HRV II. This structure was

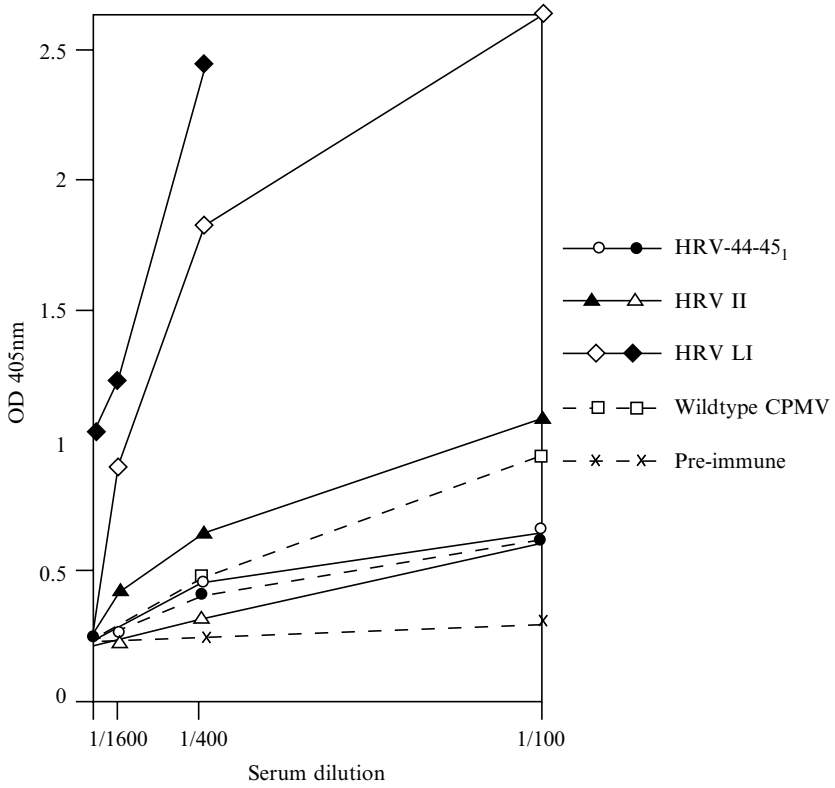


FIG 20. Recognition of HRV14 in ACP-ELISA by antisera produced in rabbits against wild-type CPMV, HRV-44-45$_1$, HRV II, and HRV L1. Reactivity of a preimmune serum is also shown. Binding was detected by the use of alkaline phosphatase-conjugated goat antirabbit antibodies and *p*-nitrophenyl phosphate. The resultant $OD_{405\ nm}$ is shown on the *y* axis. Adapted from Taylor *et al*. (2000).

determined previously (Lin *et al*., 1996). The peptide is folded as a pseudo loop bonded by a noncovalent hydrogen bond. (C) The extended conformation of the insert in CPMV/HRV-44/45$_1$. The peptide extends as far as the L subunit, and its C terminus interacts with Asn252 of the L subunit (D) Folded insertion in CPMV/HRV-44/45$_1$. The insertion folds back and interacts with the N terminus of the βC loop with its C terminus, as if the cleavage did not occur. (E) CPMV/HRV-L1. The insert is extended as far as possible, and its conformation is still unlike that in its native environment. From Taylor *et al*. (2000). (See Color Insert.)

against CPMV/HRV II and CPMV/HRV-44–45_1 chimeras bound poorly to intact HRV14 particles. Indeed, their binding curves differed little from that obtained with wild-type CPMV, suggesting that the limited binding observed might be nonspecific. However, the binding to HRV14 particles of antibodies raised against CPMV/HRV-L1 was enhanced greatly compared with that of antibodies raised against the other two CPMV/HRV chimeras. Because both CPMV/HRV II and CPMV/HRV-$44/45_1$ presented the NIm-1A site as a peptide free at its C terminus, whereas CPMV/HRV-L1 displayed it as a closed loop, these observations indicated that the structural constraint of the HRV14 peptide played an important role in its immunological properties. Despite their improved binding properties, sera raised against CPMV/HRV-L1 were like those raised against CPMV/HRV II and CPMV/HRV-$44/45_1$, nonneutralizing. This was consistent with the observation that the conformation of the inserted epitope did not adopt the native conformation.

The goal in varying the location of the HRV-specific sequence on the surface of CPMV was to determine the effect of presentation and structure on the immunogenicity of the inserted peptide. The study also revealed the effect of different sites of insertion on the extent and position of proteolytic cleavage. The fact that a number of chimeras with inserts either in the βC'-βC" loop and at various positions in the βB-βC C loop were viable indicates that CPMV particles are tolerant to the presence of foreign peptides. The observation that cleavage occurred between the two C-terminal amino acids of a heterologous sequence expressed in the βC'-βC" loop of the S protein in CPMV/HRV-$44/45_1$ suggests that position-dependent processing (Taylor *et al*., 1999a) is not a unique property of sequences expressed in the βB-βC loop. Indeed, the observation that proteolysis occurred in a sequence expressed on CPMV L protein (Brennan *et al*., 1999) indicates that cleavage of peptides on the surface of CPMV may be a general phenomenon.

This study represents the first occasion for any epitope presentation system in which the crystal structures of foreign peptides were correlated with their immunological efficacy. A previous attempt to make such a correlation, although instructive, relied on the prediction of the three-dimensional structures rather than their actual determination (Tisminetzky *et al*., 1994). Data from studies of the NIm-1A sequence presented on the CPMV surface confirm the fact that the precise mode of presentation of an epitope on the surface of a carrier can be crucial for its immunological properties. For epitopes such as NIm-1A, which adopts a constrained structure in native context, presentation as a closed loop is probably essential for good mimicry.

The fact that the structure of the NIm-1A antigenic site on HRV-14 can still not be fitted in detail to the electron density corresponding to the insert in CPMV/HRV-L1 suggests that there may be room for further improvement of presentation. Additional constraints, such as disulfide bonds and a metal-binding site, can be introduced into CPMV/HRV-L1 and the conformation of the insert can be modulated *in vitro*. Overall, results demonstrate the potential of the CPMV-based epitope presentation for studying the relationship between peptide structure and immunogenicity.

3. Generation of Effective Vaccines and Antivirals

Structural studies of CPMV chimeras with the NIm-1A epitope demonstrate the conformational requirement for an effective presentation and the potential usefulness to design vaccines by manipulation of the presentation environment. There are, however, many epitopes that do not require elaborate manipulation of the presentation to be effective. CPMV chimeras expressing epitopes from gp41 of human immunodeficiency virus type 1 (HIV-1) or VP2 of mink enteritis virus (MEV) were shown to generate neutralizing and protective antibodies, respectively, when injected into experimental animals (Dalsgaard *et al*., 1997; McLain *et al*., 1995, 1996).

CPMV chimera technology was also used for the production of a nonimmunogenic antiviral agent. Measles virus binds with high affinity to two peptides of its receptor, CD46. Engineering of one of the peptide sequences on the CPMV surface produced a chimera that inhibited the measles virus infection effectively (Khor *et al*., 2002) by competing with the cell surface for binding to the virus. It was shown that presentation on the viral surface increased the efficacy of the peptide by a factor of 200 compared to the free peptide. The measles virus protein that binds CD46 is trimeric, and presentation of the peptides with icosahedral symmetry on CPMV allows for multivalent interaction, thereby enhancing the binding.

4. Bioconjugation as a Complementary Strategy for Presentation of Proteins on the Viral Surface

There are limitations on the genetic presentation of peptides on the CPMV surface. Only peptides with moderate size, typically <40 amino acid residues, could be inserted without interfering with the virus life cycle. In many instances, even a small size did not guarantee successful presentation of a peptide by genetic means. To overcome this limitation, an alternative strategy was developed. Instead of peptide epitopes, small peptides, usually 2 to 5 amino acid residues in length

with a single Cys residue, were placed on the CPMV surface with the chimeric CPMV technology. The sulfhydryl groups inserted were chemically reactive (Wang *et al*., 2002), allowing attachment of polypeptides by bioconjugation. This is, in some respects, a return to the traditional approaches of attaching peptides to larger carrier molecules to increase their immunogenic efficacy. However, the chemical attachment of polypeptides to CPMV is site specific. Presentation of molecules conforming to viral symmetry can enhance their efficacy, as shown in the presentation of antiviral peptides against measles virus (Khor *et al*., 2002). Moreover, structural information of the target proteins can also be extracted from the virus/protein conjugate by either X-ray crystallography or cryoelectron microscopy.

A prototypic experiment of attaching peptides to the virus by bioconjugation was carried out with an A Kinase Anchoring Protein (AKAP)-derived peptide that could serve as the anchoring point for other proteins. AKAPs are a family of proteins functionally defined for their ability to bind and target cAMP-dependent protein kinase (PKA) to various subcellular locations (Fig. 21A) (Colledge and Scott,

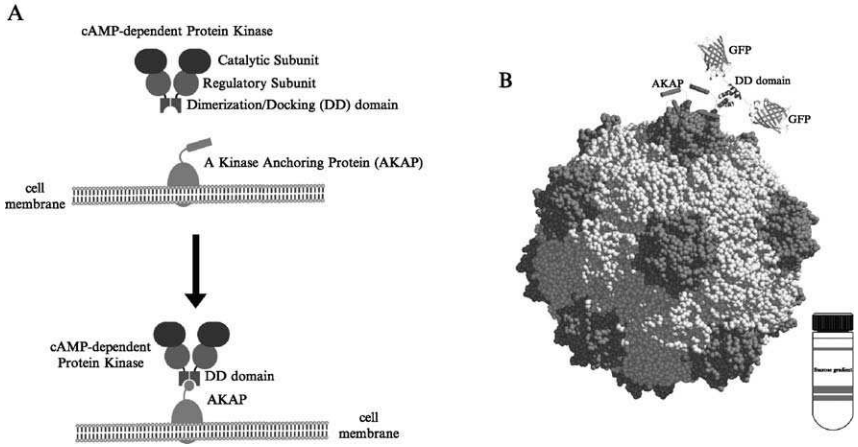


FIG 21. (A) Schematic diagram of binding of cAMP-dependent protein kinase to A kinase anchoring protein. The kinase is composed of dimmers of catalytic and regulator subunits. The dimerization/docking domain recognizes the C-terminal 27 residues of AKAP. Attachment of this 27 peptide to CPMV allows the anchoring of proteins fused with the DD domain to the virus surface. (B) Model for presenting green fluorescence protein (GFP) (in ribbon drawing) on CPMV (in space-filling model) by fusion of the protein to the DD domain and binding to the AKAP peptide (drawn as cylinders). (See Color Insert.)

1999). AKAPs contain a domain targeting the AKAP to specific subcellular locations and an A kinase-binding (AKB) domain that binds to the regulatory subunit of PKA.

The 27 residue AKB domain from D-AKAP2, an AKAP with dual specificity (i.e., being able to bind both regulatory subunit isoforms of PKA), was chosen for insertion to the capsid with chimeric CPMV technology. Virus with the peptide, however, was not viable, possibly because of the ubiquitous physiological functionality of the sequence. The peptide was then presented on the virus surface by the bioconjugation method (Chatterji *et al*., 2002). The attachment of the high-affinity-binding D-AKAP2 peptide on the viral surface will make CPMV into a general purpose attachment template, as many proteins fused with its cognate-binding domain can be presented (Fig. 21B).

IV. Assembly-Dependent Folding of Protein and RNA in Bean Pod Mottle Virus

BPMV shares similar overall genome organization, replication strategy, and capsid structure with the type member CPMV (Lin *et al*., 1999). Structural studies of BPMV provided the first observation of a portion of a viral genome in association with its capsid (Chen *et al*., 1989), and the structural comparison of a nucleoprotein particle with an empty capsid that demonstrated ordering of elements of the capsid protein depended on the binding of RNA (Lin *et al*., 2003).

A. Structures of Nucleoprotein Components

BPMV particles are separated into three components on a sucrose gradient. The bottom (B) and middle (M) components are the nucleoprotein particles, containing RNA1 and RNA2, respectively (Lomonossoff and Johnson, 1991). The top (T) component is a capsid with no RNA.

Structures of the B and M components were virtually identical, despite the different RNA content. The overall capsid structure of the BPMV nucleoprotein was similar to that of CPMV, except conformation of the N terminus of the S subunit (described in Section V). However, unlike the structure of CPMV, as well as that of all the other picorna-like viruses, six well-defined ribonucleotides (labeled NA1 to NA6) of the single-stranded RNA genome occupied the large cleft formed between the B5 and C domains that comprised a single polypeptide chain (Figs. 22 and 23). The electron density for the RNA was comparable in

FIG 22. Ribbon diagram of the BPMV asymmetric unit from the exterior. The N-terminal portion of the C2 domain (twofold-related C domain) is also shown in cyan. The N terminus of the C2 domain is sandwiched between the ordered RNA and the C domain. The ordered RNA is drawn as a ball and stick in black. The oval represents twofold symmetry. Adapted from Lin *et al*. (2003). (See Color Insert.)

quality but not connected to the protein capsid. With clearly identified positions for phosphate, sugar, and the bases, the polarity of the RNA was defined. The 5′ end of the ribonucleotide extended toward the A subunit, whereas the 3′ end extended around the threefold axis. The height of the density indicated nearly full occupancy of the 60 equivalent positions. The electron density was consistent with a nonrandom sequence of bound nucleotides. NA1 was probably an adenine, based on its density and interactions with water molecules, whereas NA2 was a purine. Pyrimidine rings readily accounted for the base density of NA3 to NA5. The base density in position 6 was not well defined and could only accommodate a five member ring (Fig. 23). The nucleotide sequence consistent with the electron density was APuPyPyPyX. The interaction of this nucleotide sequence with the capsid appeared specific and was reminiscent of the structure of bacteriophage MS2 in association with its operator (Valegard *et al*., 1994). There was a distinction, however, as the MS2 operator complex was generated by diffusion of the specific oligonucleotide of the operator sequence into virus-like particles and the same oligonucleotide occupied all 60 equivalent positions in the capsid. In native MS2, however, only 1 of the 60 positions was occupied. The presence of a dominant nucleotide

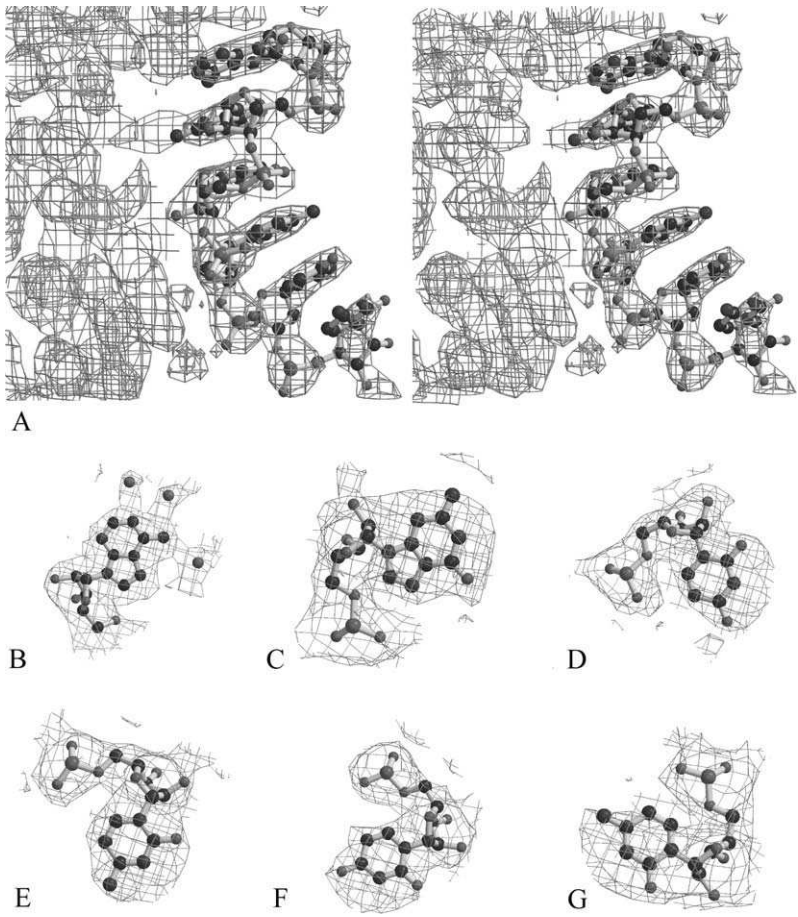


FIG 23. Electron density for RNA. (A) Stereo view of the M component. The ordered RNA model is a ball and stick. Carbon atoms are in black, nitrogen atoms are in blue, oxygen atoms are in red, and phosphate atoms are in magenta. The model of protein is not drawn. The density for the B component is very similar to that of the M component. (B) Electron density of the first nucleotide and interacting water molecules, which is best modeled as adenosine. (C) Electron density of the second base modeled as guanosine. (D–G) The third to sixth nucleotides modeled as uridine and cytosines. The electron densities are contoured at 1σ. From Lin *et al*. (2003). (See Color Insert.)

sequence visible in the electron density of BPMV suggests a consensus sequence distributed in the viral genome and that this repeating sequence may serve as the encapsidation signal for RNA packaging and assembly.

The nucleotide strand was helical with base stacking, despite being single stranded. There were many interactions with the capsid as shown in Fig. 24 and listed in Table IV. The bases of the first two nucleotides, NA1 and NA2, interacted predominantly with the capsid

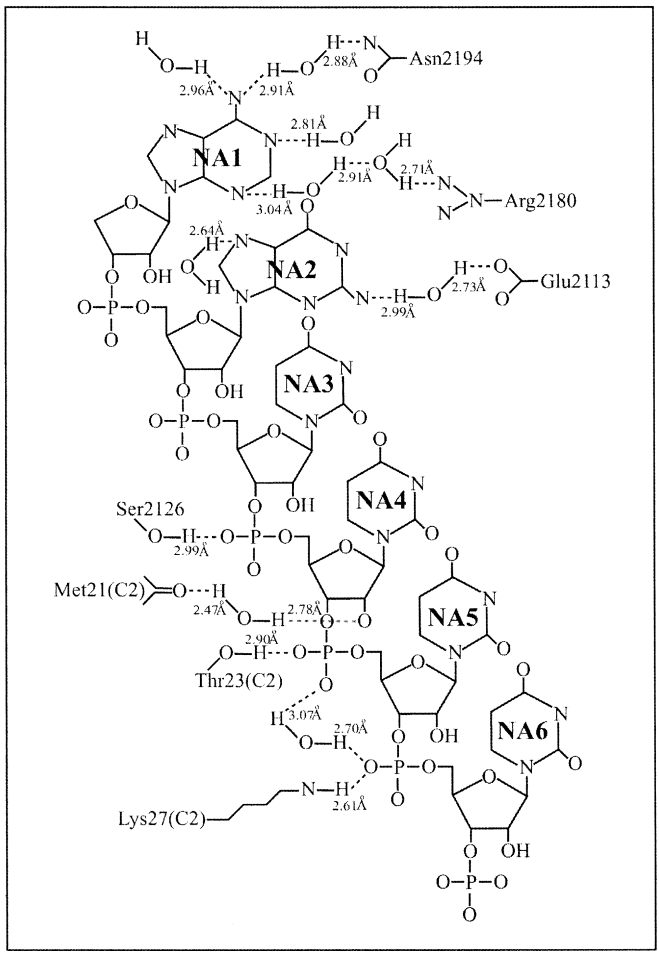


FIG 24. Interactions of the ordered RNA with the protein capsid. Bases of the first two nucleotides point toward the capsid and interact with the capsid through water molecules. Bases of the fourth, fifth, and sixth nucleotides point toward the interior and interact with residues from the C2 domain with ribose and phosphate moieties. The first two nucleotides appear to define specific interactions with the capsid protein. The nonspecific interactions of NA4 to NA6 mostly involve with the N terminus of the C2 domain. C2 indicates that the residues are from the twofold-related *C* domain. From Lin *et al*. (2003).

protein, although water molecules mediated many of these interactions. These contacts appeared to define the specificity of the binding, whereas ribose and phosphate moieties of the last three nucleotides contributed to the overall stability of the complex. The interactions of NA4–NA6 were mainly associated with the N terminus of the twofold-related L subunit (Figs. 22 and 23). This interaction apparently ordered the first 19 residues the N terminus of the L subunit, as shown in the structure of the T component, which contained no RNA (see later).

The overall backbone stereochemistry of the six ribonucleotides approximated that found in one strand of an A-type RNA duplex. The details of the geometry though were different. The angles and rises between nucleotides are listed in Table V. Of note were the angles

TABLE IV
INTERACTIONS BETWEEN RNA AND PROTEIN CAPSID

Nucleotide	Interacting group	Dist	Interacting group	Residue or water
NA1 (A)	N1 (base)	2.8	O	H_2O4112
NA1 (A)	N3 (base)	3.1	O	H_2O4187
NA1 (A)	N6 (base)	2.9	O	H_2O4113
NA1 (A)	N6 (base)	3.0	O	H_2O4114
NA2 (G)	N2 (base)	3.0	O	H_2O478
NA2 (G)	N7 (base)	2.6	O	H_2O4185
NA4	O2P (phos)	2.8	Oγ	Ser2126
NA4	O2' (ribose)	2.8	O	H_2O4146
NA5	O2P (phos)	2.9	Oγ	Thr23 (C2)
NA5	O1P (phos)	3.1	O	H_2O4145
NA6	O1P (phos)	2.6	Nζ	Lys27 (C2)
NA6	O1P (phos)	2.7	O	H_2O414

TABLE V
GEOMETRIES OF THE ORDERED NUCLEOTIDES

	Rise (Å)	Angle (°)
NA1-NA2	3.5	59
NA2-NA3	3.5	35
NA3-NA4	3.6	45
NA4-NA5	3.8	45
NA5-NA6	3.6	45

between NA1 and NA2 (59°) and between NA2 and NA3 (35°). The rest of the helical twist angles for the polyribonucleotide were 45°. The rise per residue was about 3.5 Å. In comparison with an A-type RNA helix, which had a twist angle of ~30° and a rise per residue of 2.6 Å, the single strand of viral RNA was wound tighter. The helical repeat distance of this RNA was roughly 28 Å, which was nearly the same as observed in the A-type helix. The comparable helical repeat of the BPMV nucleotide to the A-type RNA in the presence of the sharper turns between nucleotides was attributed to an overall bend in the BPMV RNA molecule (Figs. 22 and 23) in which it was stretched at the phosphate backbone and compressed at the stacking side of the bases.

The quality of the electron density for the RNA was comparable to the density for protein. The temperature factors for the bases though were roughly 45. Five more nucleotides (NA1′ to NA5′) were added to the model with reduced confidence due to the lower height of the electron density. With the exception of NA1′, however, the fit was ambiguous. Including these less defined ribonucleotides, closed an RNA trefoil ring around the three fold particle axes, each consisting of 33 ribonucleotides (Fig. 25). As viewed from outside the particle, the polarity of the chain went from 5′ to 3′ in a clockwise direction. This cluster probably represented loops in the RNA secondary structure that recognize threefold protein sites in the assembling virus. The detailed image of the RNA molecule entering and leaving the trefoils and the connections between trefoils were not observed because they did not obey icosahedral symmetry. These end effects (entering and leaving the trefoils) gave rise to the weaker RNA density in the connecting regions because only two of the three regions averaged at most were actually occupied in any given trefoil. The total RNA modeled accounted for roughly 660 ribonucleotides for the entire particle, which was about 20% of the package RNA in the M component. The similarity in the bound RNA segment between the B and M components suggested that RNA1 and RNA2 shared similar RNA packaging signals.

B. Structure of T Component and Elements Affecting the Binding of RNA

Empty BPMV capsids provide a novel feature for the study of protein RNA interactions in this system. Because the empty capsid crystallized isomorphously with the nucleoprotein components of BPMV, the structures were directly compared with difference Fourier analysis. The $(F_{mid}\text{-}F_{empty})e^{i\alpha mid}$ map showed that a significant change occurred

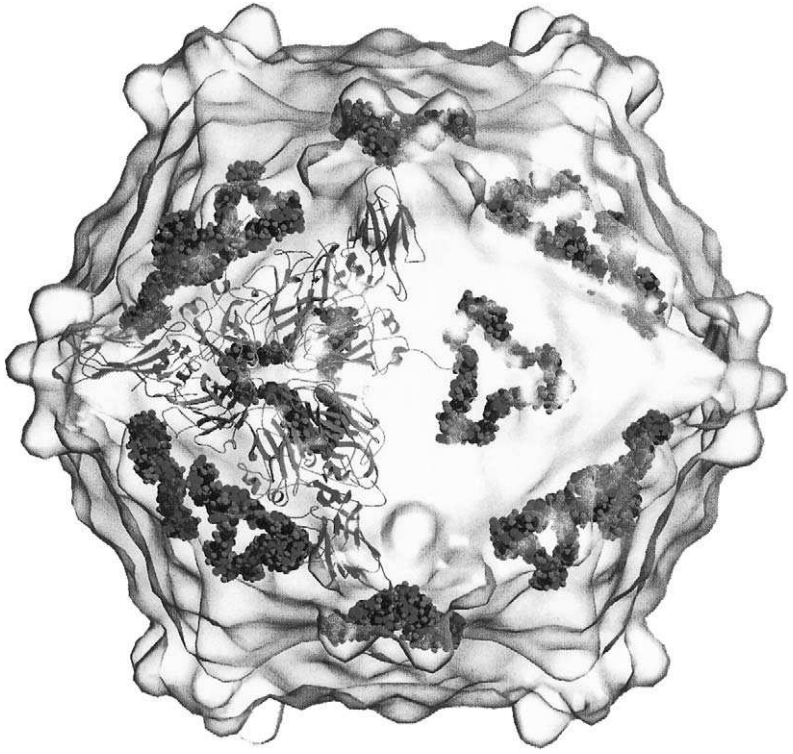


FIG 25. RNA in the viral capsid. Trefoils of RNA superimposed on the electron density viewed from the exterior. The electron density in gray scale and semitransparent was calculated to 20 Å. RNA trimers are drawn as CPK models. Carbon atoms are in black, oxygen and phosphate atoms are in red, and nitrogen atoms are in blue. The ribbon diagram of one of the protein trimers is also shown. From Lin *et al*. (2003). (See Color Insert.)

in the capsid protein when it interacted with RNA. The density in the difference map corresponded to the N terminus of the L subunit of the C domain (Fig. 26). This was the only nonmodular element in the subunit interactions (Fig. 22). This portion extended toward the neighboring B domain to reach the cleft and formed interactions with the bound RNA and protein in the two fold-related C2 domain. The ordered RNA therefore interacted directly with the twofold-related N terminus of the L subunit from the C2 domain. To investigate the conformation of this peptide in the empty particle, the atomic model without the N terminus of the L subunit was used for calculating the initial phases for

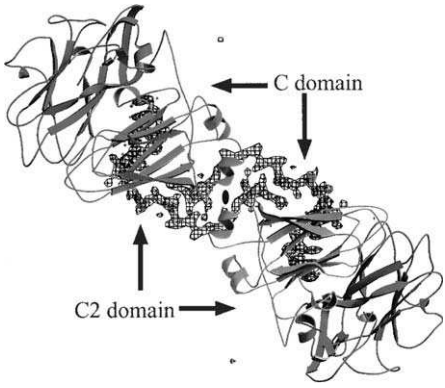


FIG 26. Difference electron density computed with $(F_M - F_T)e^{i\alpha(ave)}$ as the coefficients for Fourier synthesis. The twofold-related dimmers of RNA, B, and C domains are superimposed with the difference electron density map. The N terminus of the C domain is in interaction with the twofold-related RNA. The oval represents twofold symmetry. The reference C domain is drawn in green, whereas the twofold related C domain (C2) is in cyan. Arrows indicate the main body of the C domains and their N termini in association with electron density. From Lin *et al*. (2003). (See Color Insert.)

phase refinement. Electron density at a much lower contour was observed for the peptide, suggesting that some peptides still adopted the conformation as found in M and B components but most did not conform to the capsid symmetry.

It was demonstrated that the nucleoprotein complexes of comovirus capsids were more stable than empty capsids (Da Poian *et al*., 1994, 2002). It was also shown that $T = 3$ virus capsids in which the N-terminal arms invaded two fold-related subunits (e.g., rice yellow mottle virus) were more stable than particles in which these arms folded back into the same subunit (e.g., SCPMV) (Qu *et al*., 2000). It is clear that ordering of the N terminus of the C domain through the protein–RNA interaction in the formation of the only nonmodular interaction between the subunits is a significant factor in the stability of BPMV nucleoprotein component structures.

C. Mode of RNA Encapsidation

RNA segments binding to the capsid are likely to be discontinuous in the RNA molecule. With a defined nucleotide sequence, it is possible that these nucleotides interact with the capsid with specificity and serve as the signals for RNA packaging. The existence of an empty

capsid demonstrates that capsid proteins can assemble without RNA and, unlike some $T = 3$ viruses [e.g., some insect viruses (Fisher and Johnson, 1993; Tang *et al*., 2001)], nucleic acid does not play an essential role in the assembly process. The RNA genome depends on signals to make it the packaging target. A working hypothesis, under further investigation, proposes that RNA, free of the ribosome, is folded with signals loosely conforming to separation distances appropriate for icosahedral symmetry and that the RNA is attracted to the capsid proteins that assemble around it (Fig. 27). There are many sequences of APuPyPyPy in the BPMV genome. In RNA1 there are 64 AAPyPyPy and 43 AGPyPyPy (Di *et al*., 1999), and there are 55 AAPyPyPy and 23 AGPyPyPy in RNA2 (MacFarlane *et al*., 1991).

It is clear that binding of RNA introduces invasive interactions between icosahedral asymmetric units as the RNA molecule threads through the domains (Fig. 27B). Moreover, nucleotide binding in the pockets between C and B5 domains establishes interactions with the N terminus of the C2 domain, which induces additional nonmodular interactions and leads to strengthening of the virus capsid.

V. Red Clover Mottle Virus (RCMV): A Variation to CPMV

Based on sequence alignment, comoviruses were divided into-two subgroups: one typified by CPMV and the other by BPMV (Chen and Bruening, 1992). However, sequence alone may not be sufficient to assign other comoviruses to the individual subgroups. Additional structural "data points" for comovirus structures expand the criteria for assignment. RCMV is another member of the *comovirus* family that was investigated biochemically and genetically and its structure was determined for comparative studies (Lin *et al*., 2000).

A. Structure and Sequence-Based Fingerprints for Subtyping the Comoviruses

A noticeable surface feature of RCMV, and other comoviruses, is protrusions at the fivefold axes (Fig. 4A). This protrusion is formed by pentameric S subunits that align the long axes of their β sandwich domains nearly parallel to the adjacent fivefold axes and therefore radial to the sphere defining the particle shape. Looking down the fivefold axis toward the viral interior, there is no part of the S subunit that approached the fivefold symmetry axis generating a channel

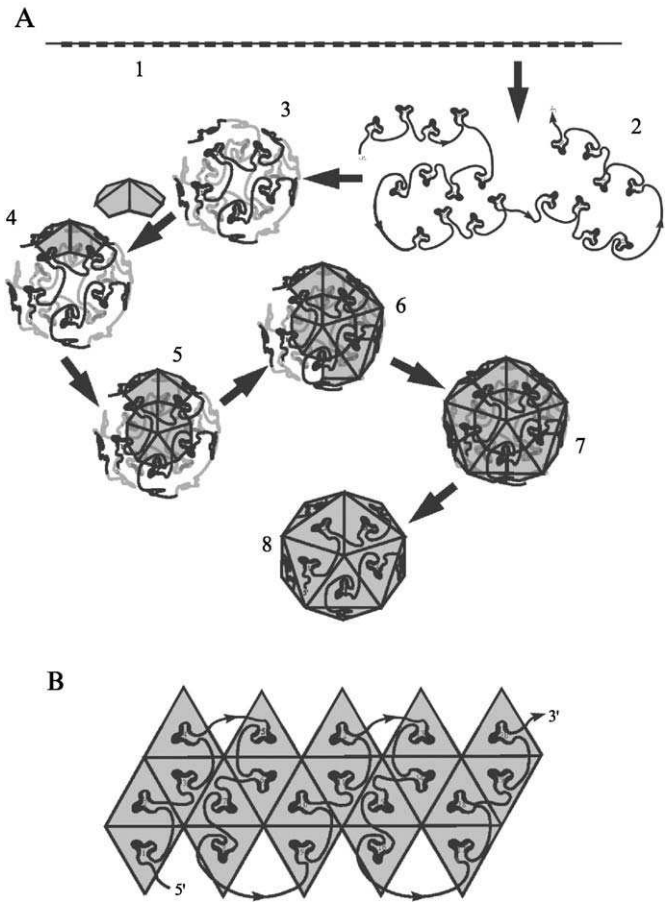


FIG 27. A proposed mode for the RNA encapsidation. (A, 1) The encapsidation signals are distributed throughout the genome. (2–3) For encapsidation, the RNA partially fold with interacting segments compatible with the icosahedral capsid. (4–8) The capsid proteins assemble around the RNA by recognition of the specific segment to form virus particles. Based on the intermediates found in poliovirus (Watanabe *et al*., 1965), pentameric capsid proteins are suggested to be the assembly unit. For clarity, only the icosahedral surface lattice and RNA in the front are shown schematically in an assembled virus particle in 8. (B) Opening of the capsid showing a thread of the RNA through the capsid that is consistent with the encapsidation model. From Lin *et al*. (2003).

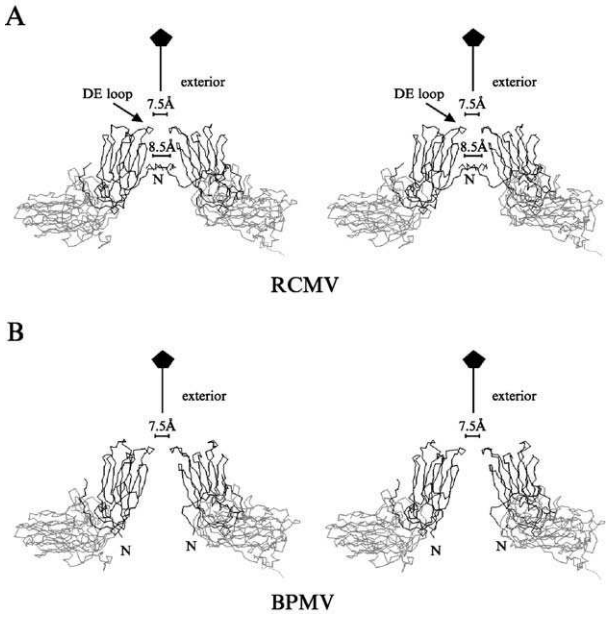


FIG 28. Stereo view of comovirus subunits in formation of a channel along the fivefold symmetry axis. The S subunit is in dark gray tracing; the L subunit is in light gray tracing. (A) RCMV. Tracings of three N-terminal residues from all five S subunits, which form the pentameric annulus, are shown. For clarity, however, only tracings of two subunits related by 144° around the viral fivefold axis are drawn. The narrowest opening located on the surface has a diameter of about 7.5 Å and is composed of the DE loops of the S subunits. The second constriction (~8.5 Å in diameter) is roughly in the middle of the channel and is formed by the pentameric annulus. A similar annulus is also identified in the CPMV structure. (B) BPMV. Two subunits related by 144° around the viral fivefold axis are shown. The N termini fold in the opposite direction to that of CPMV and there is no annular structure. From Lin *et al*. (2000).

along the fivefold axis running from the exterior to the interior (Fig. 28). RCMV bears more similarity to CPMV than to BPMV in this part of the structure.

The channel has an overall funnel shape with the narrow end at the outer surface and the wider end in the interior. The narrow end is composed by the βD-βE loops of the S subunits reaching over the innermost βF-βG loop of the β sandwich structure and clustering around the fivefold axis (Fig. 28A). The opening at this end is about 7.5 Å. Further down the fivefold axis, a second constriction is found in RCMV and CPMV, but not in BPMV (Fig. 28). This is the result of the three N-terminal residues of the S subunits forming a pentameric annulus

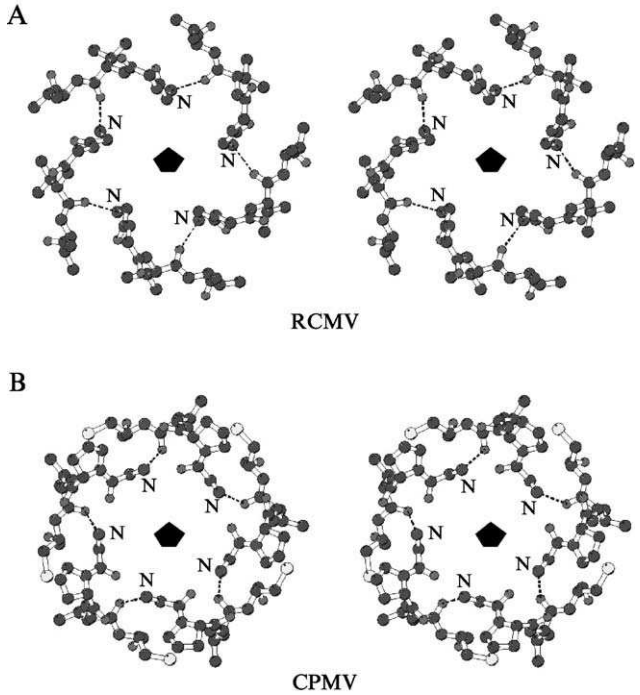


FIG 29. Stereo views of the pentameric annuli of comoviruses looking down the fivefold axis from the viral exterior. All atoms are drawn as black spheres, except the sulfur atoms, which are gray spheres. Hydrogen bonds are shown as dashedlines. (A) The pentameric annulus of RCMV. It is formed by hydrogen bonding with the first three N-terminal residues from each pentameric S subunit. The amino group of each N terminus forms a hydrogen bond with the main chain carbonyl oxygen of the neighboring third residue. The annulus is star shaped with each peptide traced upward and then downward. The opening is about 8.5 Å. (B) The pentameric annulus of CPMV. It adopts a similar hydrogen-bonding pattern to that found in RCMV. Each N terminus traces upward toward the exterior and the annulus is crown shaped. From Lin *et al*. (2000).

structure (Fig. 29). Similar annular structures were found in a number of icosahedral viruses (Hogle *et al*., 1985). In CPMV and RCMV, the amino group of the N terminus forms a hydrogen bond with the main chain carbonyl oxygen of the neighboring third residue. The opening is about 8.5 Å. No constriction was found in BPMV as it lacked the pentameric annuli (Fig. 28B).

The original subgrouping of RCMV with CPMV and BPMV with CPSMV was based on the degree of similarity between the proteins (48K, L and S) encoded by their RNA2 molecules (Chen and Bruening,

1992). However, the extensions of this approach to other comoviruses were not particularly revealing and no obvious discriminating criteria could be derived for subgrouping andean potato mottle virus (APMV) (Shindo *et al*., 1993) and several strains of squash mosaic viruses (SqMV) (Haudenshield and Palukaitis, 1998; Hu *et al*., 1993). The results were especially ambiguous in the case of APMV, which shared a high degree of similarity with BPMV but a comparatively low degree of similarity with CPSMV (Haudenshield and Palukaitis, 1998). This discrepancy was inconsistent with the previous placement of BPMV and CPSMV in the same subgroup (Chen and Bruening, 1992).

Structural alignment (i.e., protein sequence alignment based on superposition of three-dimensional structures) of RCMV, CPMV, and BPMV indicated that one of the discriminating sequence differences between the two subgroups was at the N termini of the S subunits. The N-terminal residue of the RCMV and CPMV S subunits was Gly in both cases instead of Ser as found in BPMV. Moreover, the N-terminal sequences of both the CPMV and RCMV S proteins were three residues longer than that of the corresponding BPMV protein (Fig. 30). These three residues formed the pentameric annuli observed in the X-ray crystal structures. The structure indicated that glycine was preferred as the first residue to avoid space constraints in formation of the annular structure. The shorter N termini of the S subunits of BPMV prevented formation of the annulus and they folded in the opposite direction (Fig. 28). Thus the longer N termini, with a glycine as the terminal residue, might serve as a fingerprint for the subgrouping of comoviruses between CPMV-like and BPMV-like viruses.

The aforementioned fingerprint was used to verify the subgrouping of CPSMV with BPMV based on sequence homology criteria (Chen and Bruening, 1992). Although protein sequence alignments suggested that the CPSMV S subunit would adopt a similar overall architecture to those of CPMV, RCMV, and BPMV (Fig. 30), the N terminus of the CPSMV S subunits resembled that of BPMV in both length and terminal residue. It was therefore reasonable to suggest that, as in the case of BPMV, a pentameric annular structure was not formed, supporting the previous subgrouping of CPSMV with BPMV. When the structural alignments were extended to include sequences of the S proteins of APMV (Shindo *et al*., 1993) and SqMV (Haudenshield and Palukaitis, 1998; Hu *et al*., 1993), subgrouping with BPMV was predicted in each case (Fig. 30). The N terminus of the APMV S subunit was two residues shorter than the length required for the formation of the annular structure. Moreover, the N-terminal residue was phenylalanine, which would not be conducive to the formation of the

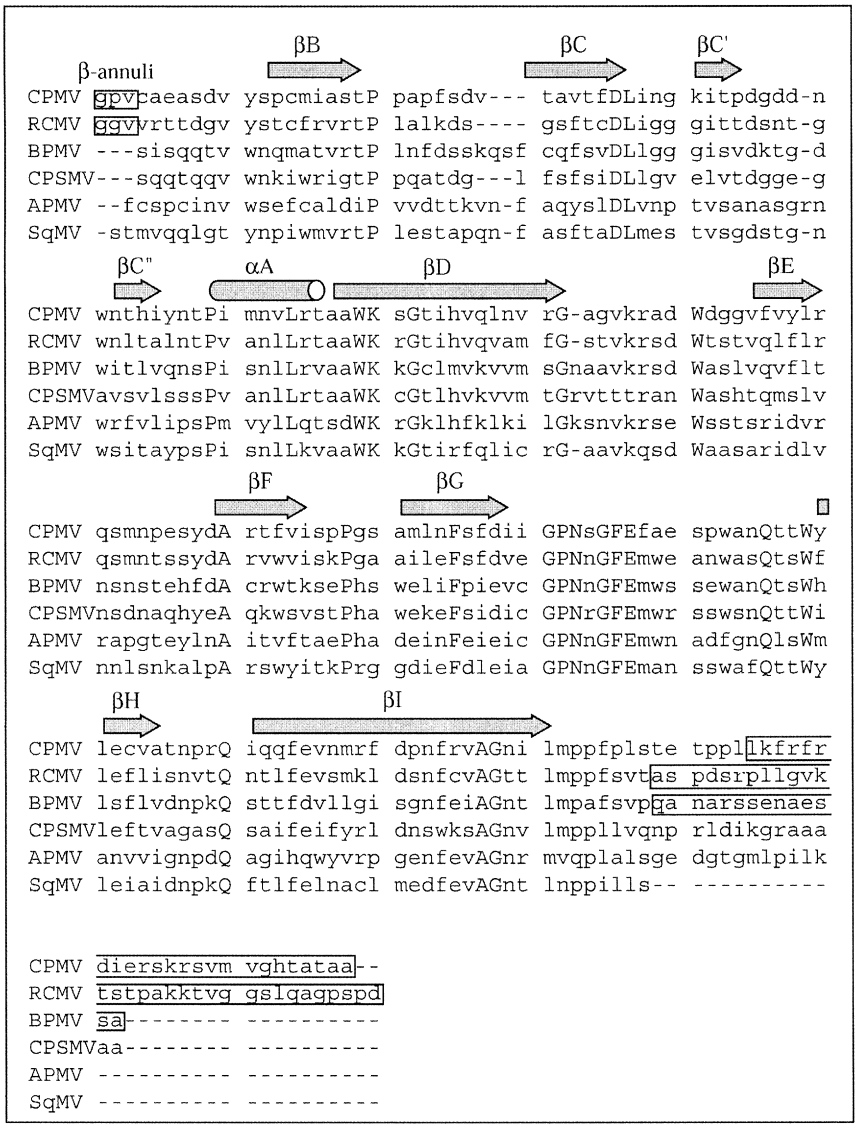


FIG 30. Sequence alignment of the comovirus S subunit based on the three-dimensional structure of RCMV, CPMV, and BPMV. The secondary structure assignments are shown at the top of the sequences. Boxed residues at the beginning of CPMV and RCMV sequence are those involved in the formation of pentameric annuli. Boxed residues in the C termini of CPMV, RCMV, and BPMV are not visible in the X-ray structure. Upper case letters represent identities. The overall similarity in the alignment suggests that all six viruses adopt similar capsid structures. The most notable

annular structure. Similarly, the N terminus of SqMV S subunit was one residue too short and, like BPMV, had a serine residue at its N terminus. It could therefore be concluded that, on structural criteria, there were two major subgroups of comoviruses. One group included CPMV and RCMV and the other included BPMV, CPSMV, APMV, and SqMV. This conclusion was consistent with the proposed subgrouping of SqMV based on the calculation of a parsimonious phylogenetic tree using RNA2 nucleotide sequences (Haudenshield and Palukaitis, 1998). Moreover, structural alignment clearly placed the AMPV in the subgroup of BPMV-like viruses.

Another noticeable feature in the structural alignment of the S proteins of different comoviruses was that the length of sequence on the C-terminal side of the βI strand for BPMV, CPSMV, APMV, and SqMV (22, 22, 20, and 8 amino acids, respectively) was considerably less for CPMV and RCMV (38 and 40 residues) (Fig. 30). Particularly striking was the fact that the C-terminal region of the SqMV S subunit was 32 residues shorter than its RCMV equivalent, having a total length equivalent to the ordered C-terminal polypeptide seen in the crystal structures of RCMV and BPMV. Although the full significance of these findings awaits further investigation, the C-terminal region of the S subunit of CPMV was implicated in the packaging of the viral RNA (Taylor *et al*., 1999b). Taking into account all data from structural and nucleotide sequence alignments, a revised scheme for subgrouping comoviruses is shown in Fig. 31.

B. Difference between Pentameric Annuli of RCMV and CPMV

Despite sharing a similar pattern of hydrogen bonding, there was a noticeable difference between the annular structures of RCMV and CPMV (Fig. 29). The annulus of RCMV was star shaped with each N-terminal sequence extending first upward and then downward. In contrast, that of CPMV was crown shaped with each N-terminal sequence extending upward toward the exterior of the virion. Of greater significance, however, was the difference in the electron densities associated with annuli in the two viruses, which seemed to critically

differences between CPMV and RCMV S subunits and those of other comoviruses reside in the N and C termini. Sequences used in the alignment are CPMV (van Wezenbeek *et al*., 1983), BPMV (MacFarlane *et al*., 1991), CPSMV (Chen and Bruening, 1992), APMV (Shindo *et al*., 1993), strain S of RCMV (Shank *et al*., 1986), and strain Z of SqMV (Haudenshield and Palukaitis, 1998). From Lin *et al*. (2000).

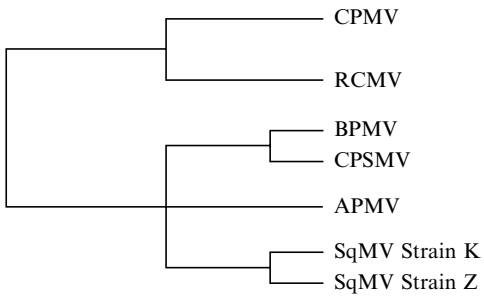


FIG 31. Subgrouping of comoviruses. Comoviruses are placed into two major subgroups, CPMV-like viruses and BPMV-like viruses, based on a discriminating structural element, the pentameric annulus. Further classification of BPMV-like viruses is based on nucleotide sequence homology calculated by Haudenshield and Palukaitis (1998). Similarity at the C termini, however, places APMV closer to BPMV and CPSMV in the BPMV-like subgroup. From Lin *et al*. (2000).

influence the annular structure. While spherical density, suggesting metal ions, was found in CPMV, much bulkier density was found in RCMV (Fig. 32). This difference was most probably due to the fact that additional purification by the CsCl gradient was employed in the preparation of CPMV for the structural studies (Lin *et al*., 1999). Conformation of the CPMV annular structure also allowed carbonyl oxygen of the N-terminal Gly to interact with the putative metal ion (Fig. 29B). The bulky density seen in RCMV probably represented some native material that could be replaced with metal ions as seen in CPMV. The location of the putative metal ion in CPMV suggested that it could diffuse further into the interior of the capsid; it was situated inside the opening of the annulus and there was no further barrier toward the interior (Fig. 32B). This was consistent with the findings that significant amounts of Cs ions diffused into the CPMV B_L component (Virudachalam *et al*., 1985) and that the annular structure was perturbed by the flux of a large amount of Cs ions into the CPMV capsid (Lin *et al*., 1999). In contrast, the bulky density found in RCMV was situated above the annulus and seemed to be prevented from further diffusion into the capsid (Fig. 32A). It seemed quite possible that the channel was a conduit, with the pentameric annulus playing a discriminatory role for the exchange of material between the viral interior and the environmental exterior. No density was found in the equivalent region of the BPMV capsid, which did not possess annuli (Chen *et al*., 1989) and was not Cs permeable.

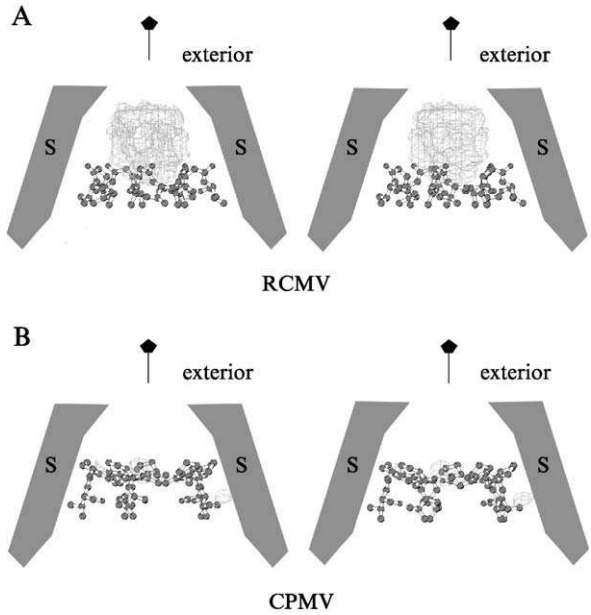


FIG 32. Stereo views of nonprotein density and pentameric annuli observed in the channel along the viral fivefold axis. Annuli are shown as ball and stick, and the electron density is drawn in chicken wire. The difference electron density maps were calculated with Fourier coefficients of $(F_o e^{i\alpha(ave)} - F_{model} e^{i\alpha(model)})$. Any densities shown in these maps were those that have not been included in the atomic model. (A) CPMV. The density is spherical, implicating metal ions, and situated inside the opening of the annulus. There is no obvious barrier between the spherical density and the viral interior. The preparation of the virus used in the structure determination involved ultracentrifugation with CsCl gradients. (B) RCMV. A much bulkier density than that in CPMV was observed. The density is atop the annulus. It appears that bulky density cannot permeate further into the capsid due to the blockage by the annular structure. The virus was prepared from infected plant leaves without ultracentrifugation in a CsCl gradient. Both electron density maps are contoured at 3.5σ. From Lin *et al*. (2000).

VI. TOBACCO RINGSPOT VIRUS: A DOWNSTREAM LINK IN EVOLUTION

Comoviridae include the plant nepoviruses and fabaviruses, as well as comoviruses (Wellink *et al*., 2000). An important consideration in the classification of comoviruses, nepoviruses, and fabaviruses in different genera is the vector of transmission. Most nepoviruses are transmitted by nematodes. Fabaviruses are transmitted by aphids, whereas comoviruses are transmitted by beetles (Wellink *et al*.,

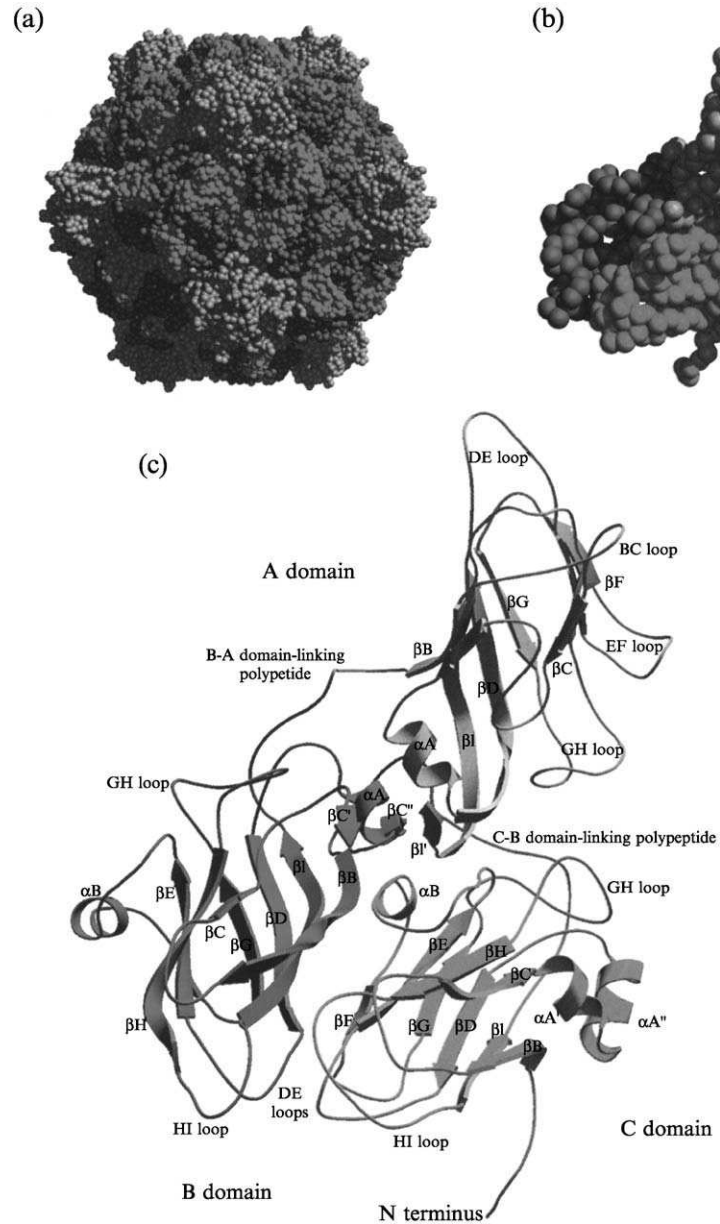


FIG 33. Structure of TRSV capsid, capsid protein, and the three domains in the capsid protein. (a) A CPK model of the $P = 3$ TRSV capsid showing the prominent surface features. The C and B domains (in green and red, respectively) are clustered around the

2000). There are also significant structural differences between comoviruses and some nepoviruses in that the capsids of comoviruses are composed of two polypeptides whereas those of nepoviruses are composed of a single polypeptide. The structure of a nepovirus, tobacco ringspot virus, was determined. Structural comparison showed that TRSV was likely to be an evolutionary intermediate of development of comoviruses (Chandrasekar and Johnson, 1998).

The TRSV capsid is composed of 60 copies of a single capsid protein (56,000 Da, 513 amino acid residues) (Buckley *et al*., 1993; Mayo *et al*., 1971). The *Nepovirus* and *Comovirus* genera in the *Comoviridae* are classified in the *picornavirus* superfamily. This classification is based on their similarity to animal picornaviruses in the translation of the genomic RNA as a polyprotein, the cleavage of this polyprotein by a virally encoded proteinase, and the sequence similarity among nonstructural proteins (Goldbach and Wellink, 1988). Previous structural studies of comoviruses and picornaviruses suggested that picorna-like viruses evolved from $T = 3$ viruses by triplication of the gene coding a β sandwich domain into a three domain polyprotein, followed by independent evolution of β sandwich domains and the development of cleavage sites in the capsid polyprotein (Chen *et al*., 1989; Hour *et al*., 1987). The picornavirus capsid polyprotein is cleaved at two sites to yield three subunits, each of which is folded into a β sandwich domain. The comovirus polyprotein, on the other hand, is cleaved at only one site to yield two subunits: one composes two β sandwich domains and the other composes one β sandwich domain. Given the size of the nepovirus capsid protein, it was hypothesized that its capsid protein might contain three covalently linked β sandwich domains and that nepoviruses might represent an example of an early stage in the development of picornaviruses (Chen *et al*., 1989).

TRSV has a similar genome organization to comoviruses (Wellink *et al*., 2000). The structure of nepovirus is also similar to comoviruses with three jelly roll β sandwich domains forming the asymmetric unit (Fig. 33) and it can be superimposed with the counterpart of

threefold axes, whereas the domains (cyan) are clustered around the fivefold axes. (b) An enlarged view of 1 of the 60 copies of the capsid protein in the TRSV capsid represented as a CPK model. (c) Representation of the tertiary structure of the C, B, and A domains in the capsid. The secondary structure featured in the three domains, the two domain-linking polypeptides, and the N and C termini of the capsid protein are indicated. From Chandrasekar and Johnson (1997). (See Color Insert.)

FIG 34. Stereo view Cα traces of the capsid proteins of TRSV superpositioned on the capsid proteins of BPMV. The superposition is viewed from outside the capsid. The C, B, and A domains in TRSV are shown in green, red, and cyan, respectively; the C and B domains of BPMV are shown in orange and the A domain is in purple. From Chandrasekar and Johnson (1997). (See Color Insert.)

comoviruses (Fig. 34). In fact, TRSV domains can be better superposed with the counterparts of comoviruses than superposition of the three domains among themselves. The most significant structural difference between TRSV and comoviruses is in the termini of the domains and the peptides connecting the domains. The connecting peptide connecting C and B domains adopts different conformations between TRSV and comoviruses. The N terminus of the TRSV capsid protein folds in a different direction compared to the counterpart in comoviruses, the N terminus of L subunits (Fig. 34).

The obvious different physical state between TRSV and comoviruses is in the coding sequence between B and A domains, which is a continuous peptide in TRSV, yet a breakage at this peptide in comovirus divides the A domain from the B and C domains into S and L subunits. The connecting peptide of B and A domains is a direct connection of a peptide without any obvious extension and unique conformation. The cleaved peptide in CPMV, on the other hand, adopts different conformations (Fig. 35). The product of the cleavage in comoviruses is the C terminus of L and the N terminus of S subunits. Unlike the TRSV

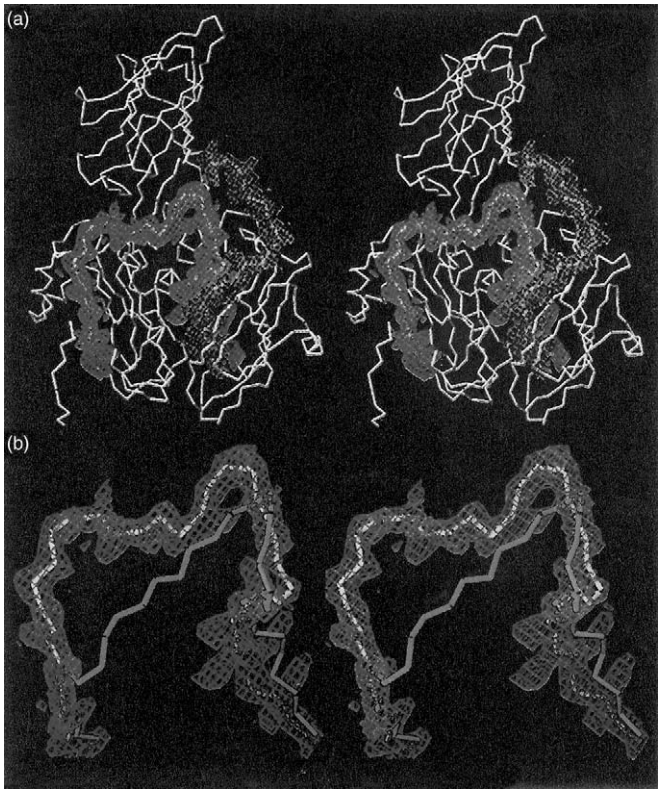


FIG 35. Interdomain linking polypeptides in TRSV. (a) Stereo view of the Cα trace (white) of the capsid protein from inside the capsid with the electron density for the two domain-linking polypeptides. The pink density shows the link between C and B domains, and the brown density shows the link between B and A domains. (b) An enlarged view of (a) showing the difference in the conformation of the C–B domain-connecting polypeptides in TRSV (yellow) and BPMV (green). From Chandrasekar and Johnson (1997). (See Color Insert.)

in which the connecting peptide is completely in the interior of the capsid, the C terminus of the CPMV L subunit threads from the interior to the exterior of the capsid between subunit contacts. The N terminus of the S subunit remains in the interior. However, a conformational difference exists between comoviruses in this structural element. The N termini of the S subunit of CPMV and RCMV fold toward the tip of the pentameric protrusion and forms annuli, whereas that of BPMV fold at the opposite direction as was discussed before. The N terminus of the S subunit of BPMV folds at

the similar direction as in the TRSV, which shows that BPMV can be evolutionarily closer than CPMV to TRSV.

The crystal structure of TRSV reveals that the capsid protein subunit is folded into three β sandwich domains that are covalently linked together by extending polypeptides as "beads on a string." The observed order of connectivity of the three domains from the N to the C terminus in the capsid protein subunit is consistent with the proposed connectivity of precleaved comovirus and picornavirus capsid polyproteins. The three different domains within TRSV and comoviruses are more closely related at the structural level than the corresponding three domains within picornaviruses. Results of structural comparison and a sequence alignment among nepoviruses and comoviruses support the notion that the capsid polyproteins of nepovirus, comovirus, and picornaviruses evolve from a common ancestor via divergent evolution. The first structure of a nepovirus also provided a snapshot of how the development of cleavage sites in the capsid polyproteins yielded new flexible N and C termini, which could intertwine and stabilize the quaternary interactions between the subunits during capsid assembly.

VII. Virus as Nanomaterials: Development of Addressable, Self-Organizing Nanoblocks with CPMV

While supramolecular chemistry and microfabrication techniques aim to converge on the scale of 1 to 100 nm by either making small molecules bigger or making big structures smaller, biology employs many constructs of the similar size. A branch of the burgeoning field of nanotechnology seeks to mimic the information-handling, materials-building, and responsive-sensing capabilities of biological systems on the nanometer scale. CPMV particles were rendered as addressable building blocks in the nanometer scale with position-controlled and programmable chemistry (Wang *et al*., 2002).

CPMV shares many notable features to be exploited for biomaterials (Whitesides *et al*., 1991), only in nanometer size. The physical, biological, and genetic properties of CPMV are well characterized (Lomonossoff and Johnson, 1991). Gram quantities of the virus are obtained routinely from a kilogram of infected black-eyed peas. The structure of CPMV is characterized to near-atomic resolution (Lin *et al*., 1999), and site-directed and insertional mutagenesis can be performed efficiently with infectious clones of the virus (Dessens and Lomonossoff, 1993; Lin *et al*., 1996; Lomonossoff and Johnson, 1996).

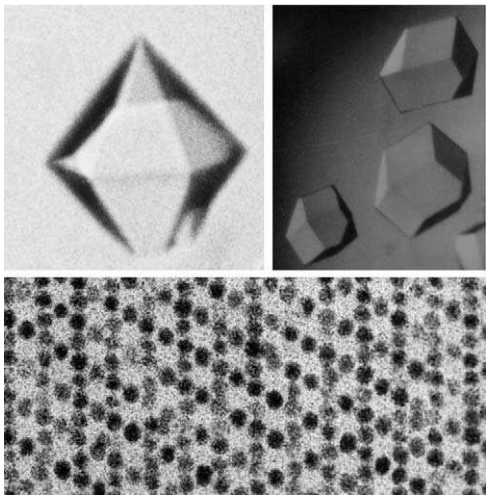


FIG 36. (Top) Hexagonal (left) and cubic (right) three-dimensional crystals of CPMV. (Bottom). Electron micrograph of crystals thin sectioned perpendicular to the *c* axis showing the open lattice. Previous studies showed that proteins with dimensions in excess of 50 Å could be soaked reversibly into the crystals. A typical crystal contained 10^{13} particles.

The particles are exceptionally stable, maintaining their integrity at 60°C (pH 7) for at least 1 h and at pH values from 3 to 9 for over a year at room temperature. Three-dimensional arrays of the virus can be readily produced under well-defined conditions (Fig. 36) (Johnson and Hollingshead, 1981; White and Johnson, 1980).

Thiol-selective chemical reagents were employed to probe the reactivity of nondisulfide-linked cysteine residues in the native virus particle. There was no free sulfhydryl on the exterior CPMV surface as shown in the crystal structure (Lin *et al.*, 1999) and no evidence of reaction with the commercially available monomaleimido-Nanogold reagent (Nanoprobes, Inc., Yaphank, NY) that had a molecular diameter of 1.4 nm. However, adducts of the native virus with ethyl mercury phosphate (EMP), an agent with a strong affinity for free sulfhydryls but with a dimension of only a fraction of a nanometer, were formed readily. The resulting labeled virus was crystallized under conditions employed previously (White and Johnson, 1980). Crystals with rhombic dodecahedral morphology, identical to those obtained with native virus, were obtained and the derivatized virus structure was determined to 6 Å resolution. Figure 37A shows

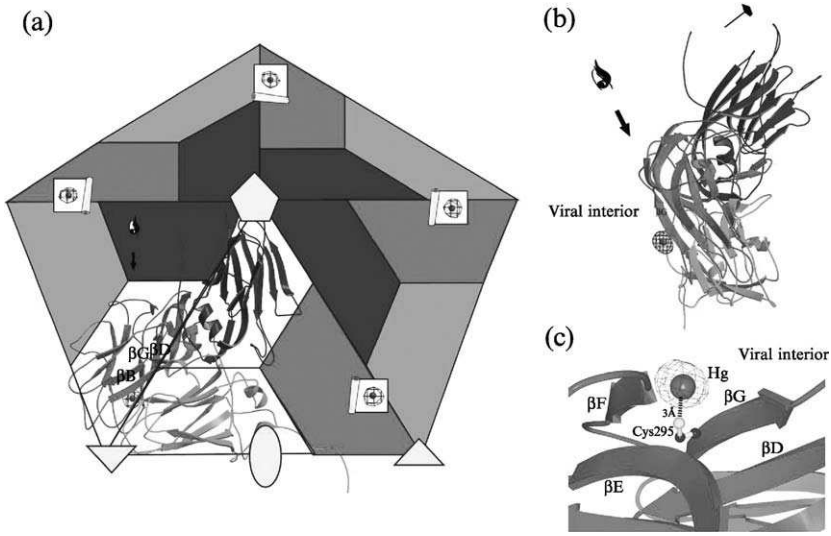


FIG 37. Crystallographic analysis of CPMV particles derivatized with ethyl mercury phosphate. Amplitudes in the Fourier series calculation were obtained by subtracting structure amplitudes computed from the atomic model of native CPMV from the measured structure amplitudes of the EMP derivative. Difference amplitudes and native phases were used to compute electron density. (a) The pentameric assembly of the CPMV protein about the fivefold symmetry axis. The difference electron density map reveals bound EMP molecules located solely at a single position below the outer capsid surface corresponding to Cys295; five such sites are shown here. (b) A view showing the fold of the CPMV asymmetric unit with EMP difference density. (c) A close-up view showing the position of the EMP difference density. From Wang *et al*. (2002). (See Color Insert.)

a pentamer of the icosahedral constellation of EMP molecules visualized in a difference electron density map. Figures 37B and 37C show that the EMP reacted with a single Cys residue at position 295, on the interior surface of the large subunit.

The reactivity of native CPMV toward an organic thiol-selective reagent was found to be different than toward mercuric ion. Thus, wild-type CPMV was condensed with 5-maleimidofluorescein (**1**); Figure 38 shows a plot of the number of molecules of dye attached to native CPMV as a function of increasing concentration of the dye reagent employed (Simeonv *et al*., 2000).

The curve plateaus at 60 attached dye molecules per particle, suggesting that a single cysteine residue per icosahedral asymmetric unit was most reactive. Denaturing gel electrophoresis analysis of the

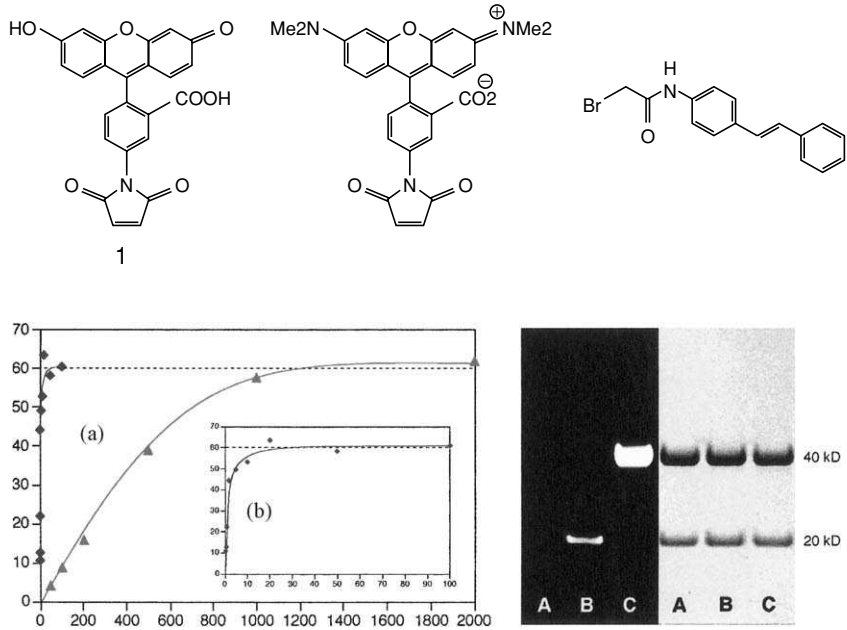


FIG 38. (Left). Plots showing the number of covalently attached fluorescein molecules to native CPMV (a) and the Cys insertional mutant virus (b) as a function of increasing ratio of reagent **1** to virus. Stoichiometries were determined by absorption measurements on solutions of labeled particles by comparison of the intensities of the dye (494 nm) to protein (280 nm), each having nonoverlapping bands with well-established molar absorptivities. Covalently modified virions were separated from excess dye reagents by multiple passages through size-exclusion filters until the dye was undetectable in the wash solution and the relevant absorbance ratios for the virus samples were constant. The intact nature of the derived particles was substantiated by sucrose gradient ultracentrifugation and size-exclusion FPLC chromatography (Superose-6). Yields of labeled virus particles were 70–80%; multiple independent experiments showed experimental error to be ±12% of the reported value for dye attachment. (Right) SDS–PAGE analysis: (A) wild-type CPMV; (B) wild-type CPMV–1 conjugate having approximately 17 dye molecules attached per capsid; and (C) mutant CPMV–1 conjugate containing 60 dyes per capsid. On the left (black background), the gel is visualized directly under ultraviolet light, showing the fluorescein emission localized in the small protein subunit of the wild-type virus and the large subunit of the mutant. On the right (light background), the gel is visualized after Coomassie blue staining, showing both small and large subunits. From Wang *et al*. (2002). (See Color Insert.)

derivatized protein showed that the dye was attached exclusively to the small subunit (Fig. 38), not to the large subunit as was the case for the reaction of EMP at Cys295. This alkylation reaction must also occur on the interior capsid surface (see below), but its exact position has not yet

been established. Multiple interior cysteine sites, on both small and large subunits, could be addressed with **1** at higher dye-to-subunit ratios without damaging the structural integrity of the particle (data not shown). Small molecules such as **1** were thus apparently able to diffuse through the capsid, perhaps through the channel that appeared in the crystal structure at each fivefold symmetry axis (Fig. 28A).

Mutant CPMV particles were prepared to display sulfhydryl groups on the exterior surface of the structure. A five residue insertion containing cysteine (GGCGG) was placed between positions 298 and 299 of the βE-βF loop in the large subunit (Fig. 12). Yields comparable to wild type were obtained with this mutant, but the presence of β-mercaptoethanol or tris(2-carboxyethyl)phosphine was required throughout the isolation procedure and in storage to avoid cross-linking of particles. These particles were reacted with increasing concentrations of fluorescent dye, with the results shown in Fig. 38. Up to 60 dye molecules were attached to each CPMV virion at significantly faster rates with smaller quantities of the dye-maleimide reagent than were observed with the native particle. The reactive interior Cys residue remained active in the mutant particle, such that the two positions could be addressed sequentially under controlled conditions. A reaction with 50 equivalents of **1**, followed by purification and reaction with 1000 equivalents of 5-maleimide tetramethylrhodamine (**2**), derivatized CPMV with an average of 55 fluoresceins and 49 rhodamines per particle, as determined by UV-VIS absorbance spectroscopy.

The differential properties of the reactive cysteine residues of the wild-type virus and the mutant virus were probed using the stilbene derivative **3** and a catalytic antibody (19G2) that produced fluorescence upon binding to stilbene (Fig. 39) (Simeonov *et al*., 2000). Because the bromoacetamide group of **3** was selectively reactive with cysteine sulfhydryl groups under conditions similar to those used for maleimides, CPMV–stilbene conjugates were prepared and purified readily for native and mutant CPMV particles. When treated with antibody 19G2, the presence of an antibody–stilbene complex was revealed by blue fluorescence upon excitation with a hand-held ultraviolet lamp; no fluorescence was observed in the absence of either stilbene or antibody. Figure 39 shows results of intact and denatured CPMV conjugates of **3**. The successful attachment of the stilbene group to both wild-type and mutant CPMV was shown by the appearance of strong fluorescence when the denatured samples were treated with the antibody. The mutant CPMV–**3** conjugate similarly showed antibody binding to stilbene on the intact particle, but the wild-type CPMV–**3**

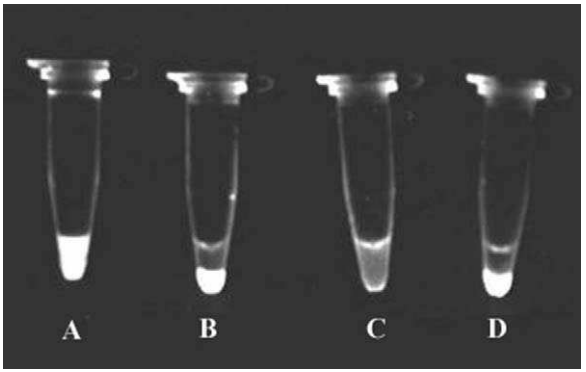


FIG 39. Samples under ultraviolet irradiation: (A) mutant CPMV–**3** conjugate + stilbene-binding antibody 19G2; (B) denatured mutant CPMV–**3** conjugate + 19G2; (C) wild-type CPMV–**3** conjugate + 19G2; and (D) denatured wild-type CPMV–**3** conjugate + 19G2. Note that denaturation causes precipitation of the viral protein, but exposed stilbenes are still recognized by the antibody. Adapted from Wang *et al*. (2002).

conjugate did not. This was consistent with the attachment of **3** to the interior capsid surface of the wild-type virus, where it was inaccessible to the indicating antibody. In contrast, the mutant virus displayed its stilbene-decorated cysteine residues to solvent on the exterior surface.

The mutant virus was also reacted with monomaleimido-Nanogold, providing virions displaying absorbance at 420 nm, indicating attachment of the gold cluster. Derivatized particles were flash frozen and examined by electron microscopy, and a three-dimensional image reconstruction was computed. Figure 40A shows reconstruction and Fig. 40B shows a difference map in which density for the model CPMV structure was subtracted from the density computed in the image reconstruction. The gold particles were clearly visible at the positions of the inserted cysteine residues (Fig. 40C), providing an example of the installation of functional structures at designated positions on the icosahedral protein template.

This is the first demonstration that a virus can function as a convenient and programmable platform for chemical reactions. Derivatized CPMV particles will generally display 60 copies of the attached molecule, making these systems analogous to very large dendrimers. High local concentrations of the attached chemical agent are thereby engineered in the vicinity of the particle, which may result in novel chemical and/or biological properties. Appropriate choices of derivatizing agents can selectively target residues on the inner or outer surface

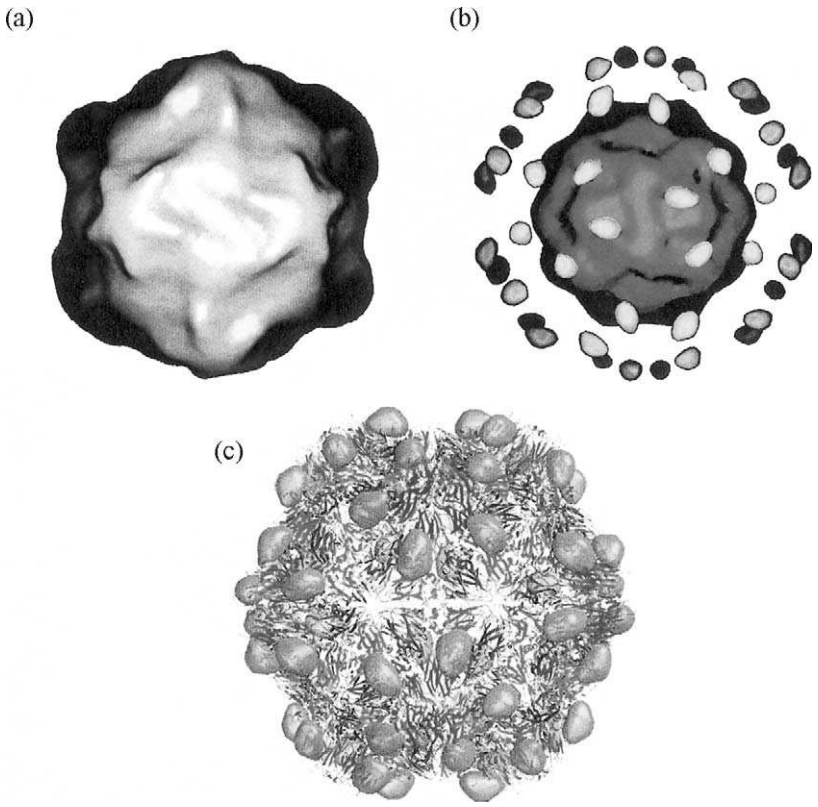


FIG 40. Electron cryomicroscopy analysis of the derivatized CPMV Cys mutant. (a) Three-dimensional reconstruction of CPMV particles at 29 Å resolution labeled with 1.4-nm gold particles. (b) A difference electron density map was generated by subtracting density computed with the native CPMV X-ray structure from the density shown in a. Because the computed native CPMV density was made from only protein, nucleic acid (shown in green) was visible in the difference map as well as the gold particles. (c) Difference electron density superimposed on the atomic model of CPMV showing that the gold is attached at the site of the Cys mutation. (See Color Insert.)

of the particle, allowing double labeling of the particles with different molecules. The range of reactivity of CPMV is being expanded with different mutations and chemical derivatizing agents, with the goal of engineering novel function within a single particle and for arrays of particles. The virus surface is patterned with metal nanoparticles with multiple cysteine residues placed at accessible positions, which can potentially be used to form a conducting "wire" at the nanometer scale.

Furthermore, CPMV icosahedra show a propensity for self-organization. Straightforward crystallization procedures led to well-ordered arrays of 10^{13} particles in a typical crystal with a size of 1 mm^3, which may be regarded as a meso scale self-organization of nanoblock components (Bowden *et al*., 2001; Breen *et al*., 1999).

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ADVANCES IN VIRUS RESEARCH, VOL 62

CUCUMOVIRUSES

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Research on the molecular biology of cucumoviruses and their plant–virus interactions has been very extensive in the last decade. Cucumovirus genome structures have been analyzed, giving new insights into their genetic variability, evolution, and taxonomy. A new viral gene has been discovered, and its role in promoting virus infection has been delineated. The localization and various functions of each viral-encoded gene product have been established. The particle structures of *Cucumber mosaic virus* (CMV) and *Tomato aspermy virus* have been determined. Pathogenicity domains have been mapped, and barriers to virus infection have been localized. The movement pathways of the viruses in some hosts have been discerned, and viral mutants affecting the movement processes have been identified. Host responses to viral infection have been characterized, both temporally and spatially. Progress has been made in determining the mechanisms of replication, gene expression, and transmission of CMV. The pathogenicity determinants of various satellite RNAs have been characterized, and the importance of secondary structure in satellite RNA-mediated interactions has been recognized. Novel plant genes specifying resistance to infection by CMV have been identified. In some cases, these genes have been mapped, and one resistance gene to CMV has been isolated and characterized. Pathogen-derived resistance has been demonstrated against CMV using various segments of the CMV genome, and the mechanisms of some of these forms of resistances have been analyzed. Finally, the nature of synergistic interactions between CMV and other viruses has been characterized. This review highlights these various achievements in the context of the previous work on the biology of cucumoviruses and their interactions with plants.

I. Introduction

Considerable advances have been made in our understanding of the molecular biology and biochemistry of the cucumoviruses since they were last reviewed in detail (Edwardson and Christie, 1991). While most of the progress concerning gene functions and molecular mechanisms has been made with *Cucumber mosaic virus* (CMV), there have also been many advances made with *Peanut stunt virus* (PSV) and *Tomato aspermy virus* (TAV). The research results and conclusions include the following: (a) The nucleotide sequences of several strains of all three viruses have been obtained and their taxonomic relationships have been clarified, if not made simpler. (b) Hybrid viruses created within and between these viral species have enabled the delineation

of the roles of gene products in various functions. (c) A new interspecies hybrid cucumovirus has been described. (d) Recombination was demonstrated to occur during cucumovirus evolution. (e) The role of recombination and pseudorecombination in CMV epidemiology has been examined. (f) Defective RNAs and new subgenomic RNAs have been identified and characterized. (g) A new gene has been discovered in the genome of cucumoviruses, and some of its functions have been determined, including a role in suppressing gene silencing. (h) The roles of various viral gene products in replication and movement have been discerned. (i) The subcellular site of CMV replication has been determined. (j) The subcellular location of the CMV movement protein has been delineated. (k) Promoter sequences for CMV replication have been mapped, as have promoter sequences for subgenomic RNA production. (l) Transgenic plants have been generated expressing various viral sequences used to engender resistance to virus infection. (m) Transgenic plants expressing viral genes have also been used for the complementation of viral gene functions or to determine viral gene functions. (n) Viral sequences involved in pathogenicity and host range have been identified and mapped. (o) The three-dimensional structures of the CMV and TAV particles have been resolved. (p) The topography of various epitopes on the surface of virions has been identified. (q) Sequences in the capsid protein important for the aphid transmission of CMV and various host interactions have been identified and mapped to the particle structure. (r) The interactions between CMV and other viruses in synergy have been characterized. (s) Progress has been made on identifying new genes for resistance to CMV and mapping such resistance genes, and one gene conferring resistance to CMV has been isolated and characterized. (t) The spatial and temporal dynamics of host enzyme activities during local virus infection has been analyzed. (u) Downregulation and upregulation of the expression of various host gene have been observed. (v) Secondary structures have been analyzed for three satellite RNAs of CMV. (w) Sequences and/or potential structures important in satellite-mediated pathogenicity have been identified.

This review describes and comments on these various discoveries and experimental observations. We will attempt to place this information into perspective, but will not again cover data reviewed previously in detail. We refer the reader to extensive reviews on the basic biology and physical properties of all three cucumoviruses (Edwardson and Christie, 1991; Kaper and Waterworth, 1981), as well as the biology and molecular biology of CMV (Palukaitis *et al*., 1992). We also refer the reader to a more extensive recent review on the functions

and properties of CMV satellite RNAs (García-Arenal and Palukaitis, 1999). We will only cover such previously reviewed information here if it is essential for introduction or if earlier conclusions are no longer believed to be correct. It is hoped that readers with varying interests will find this review useful in itself, as well as a supplement to previous reviews.

CMV, PSV, and TAV are the three viruses presently admitted as species of the genus *Cucumovirus*, in the family *Bromoviridae* (van Regenmortel *et al*., 2000). Although superfamilies have no official status in the taxonomy of plant viruses, the family *Bromoviridae* is often referred to as being in the alphavirus-like or Sindbis virus-like superfamily. Other putative members of the genus *Cucumovirus* listed in the literature include *Cowpea banding mosaic virus* (Sharma and Varma, 1975), *Cowpea ringspot virus* (Phatak, 1974; Phatak *et al*., 1976), and *Bean distortion mosaic virus* (White *et al*., 1995a). Because the first two of these viruses have not been characterized molecularly and there do not appear to be any reports concerning these viruses since they were last reviewed (Edwardson and Christie, 1991), we will not mention them further here. The last virus is a natural pseudorecombinant between CMV and TAV (see Section VII,B). Thus, this review is confined to CMV, PSV, and TAV, with most of the data coming from CMV.

II. Particle and Genome Structure

A. *Particle Structure*

Cucumoviruses have icosahedral particles with a $T = 3$ quasisymmetry (Finch *et al*., 1967; Francki *et al*., 1966). Particles are 29 nm in diameter, have 180 capsid protein subunits, and consist of about 18% RNA (Finch *et al*., 1967; Francki and Hatta, 1980; Habili and Francki, 1974a; Kaper and Re, 1974). Particles of CMV and TAV (Habili and Francki, 1974a), or of all three cucumoviruses (Tolin, 1977), are indistinguishable morphologically and have a similar physical and chemical composition. CMV and TAV particles differ in their stability after exposure to magnesium ions (Habili and Francki, 1974b). Cucumoviral particles are stabilized by RNA–protein interactions and are disrupted easily at a high concentration of neutral chloride salts or in the presence of sodium dodecyl sulfate (Kaper, 1975). Biologically active particles can be reassembled readily from RNA and soluble capsid protein by lowering the salt concentration or removing the

sodium dodecyl sulfate (Kaper, 1969). In contrast to bromoviruses, which have similar and well-characterized particles, cucumoviruses do not form empty particles *in vitro*, do not swell at pH 7.0, and are stable at pH 9.0 (Smith *et al*., 2000).

The structures of TAV and CMV particles have been analyzed by high-resolution X-ray crystallography (Canady *et al*., 1995; Lucas *et al*., 2002; Smith *et al*., 2000; Wikoff *et al*., 1997). For CMV, structure analysis has been carried out at 3.2 Å resolution. Analysis showed that the subunits form several arrangements with axes of twofold, threefold, fivefold, quasi-threefold, and quasi-sixfold symmetry. The exterior radius along the quasi-sixfold axes is 144 Å, and the density along the fivefold axes extends 3 Å more. The RNA is tightly packed against the protein shell, leaving a hollow core of about 110 Å along the threefold axes. The electron density map of the protein subunit is consistent with a β-barrel structure, with the long axis of the β-barrel domain oriented in a roughly radial direction, as is also the case for the *Bromovirus Cowpea chlorotic mottle virus* (Speir *et al*., 1995), which has very similar particles (Smith *et al*., 2000).

The N-terminal 22 amino acid residues of the capsid protein have a high density of arginine residues, a net positive charge of +7, and probably interact with the RNA. This arginine-rich region is necessary for particle formation (Schmitz and Rao, 1998). A unique feature of CMV as compared with other $T = 3$ plant viruses is that N termini of the B and C subunits form a hexameric bundle of amphipathic helices that runs parallel to the quasi-sixfold axes, starting at ~85 Å from the center of the virion and extending to the capsid–RNA interface. One site of the capsid, in an external loop between β strands H and I (the H-I loop), the sequence of which is conserved among cucumoviruses, could be involved in metal interactions (Smith *et al*., 2000). This loop is highly antigenic (He *et al*., 1998) and is involved in aphid transmission (see Section IV,C). A monoclonal antibody specific to this loop was found to bind only to the pentameric clusters of the A subunits and not to the same sequences present in the hexameric clusters of the B and C subunits, demonstrating differences in quasiequivalence (Bowman *et al*., 2002).

The structure of TAV particles has been analyzed with an effective resolution of 4 Å (Lucas *et al*., 2002). The TAV structure is very similar to that of CMV. Major differences in the α-helical loop between β strands E and F (the E-F loop), which is exposed to the exterior environment of the virus and could confer host or vector specificities unique to each virus. Differences also occur in the F-G loop of the A subunits, resulting in smaller pore sizes at the fivefold axes of TAV, which could

explain the greater resistance of TAV compared with CMV to RNase (Habili and Francki, 1974a,b). In addition, disulfide bonds are formed between Cys-69 and Cys-106 (Arg-106 in CMV), a further difference between the two viruses. The similarity of the three-dimensional structure of CMV and TAV capsids agrees and explains the observation that stable particles can be assembled *in vitro* from capsid protein subunits of either virus (Chen *et al*., 1995). Moreover, recombinant viruses with chimeric CMV/TAV capsid proteins are infectious and form stable particles (I. Moreno and F. García-Arenal, unpublished results).

Although of similar morphology and sedimentation properties, cucumovirus particles differ in encapsidated RNA: RNAs 1 and 2 are encapsidated separately, whereas RNA 3 and the subgenomic RNA 4 are probably encapsidated in the same particle (Habili and Francki, 1974a; Lot and Kaper, 1976). Particles with three copies of RNA 4 may also exist (Kaplan and Palukaitis, 1998). Although the CMV capsid protein can assemble into particles using different RNAs *in vitro* (Kaper and Geelen, 1971; Chen and Francki, 1990), indicating a lack of rigorous sequence specificity, evidence shows that this is not the case *in vivo*. In a hybrid virus formed between CMV and the *Bromovirus Brome mosaic virus* (BMV), the CMV capsid protein could encapsidate BMV RNAs 3 and 4 with a low efficiency *in vivo* (Osman *et al*., 1998). There is also a limit to the size of the encapsidated RNA, with RNAs larger than RNA 1 not encapsidated *in vivo* (Palukaitis and Zhang, 1996).

B. Genome Structure

The genome of cucumoviruses consists of three single-stranded messenger sense RNAs, named 1, 2, and 3, in order of decreasing size (Fig. 1). All three genomic RNAs of isolates of the same species could be reassorted into new functional isolates; however, only RNA 3 could be exchanged between different species (Rao and Francki, 1981). On the other hand, it has been shown that a mixture of RNA 2 of both CMV and TAV can be maintained stably in infected plants by TAV RNA 1 and CMV RNA 3 (Masuta *et al*., 1998). Each of the CMV RNAs has a 5′-cap structure (Symons, 1975) and a 3′ terminus that can adopt a pseudoknot structure similar to that of a tRNA (Rietveld *et al*., 1983). This 3′-terminal structure was predicted to be different for CMV and TAV (Joshi *et al*., 1983). While the 3′ ends of CMV RNAs could be specifically aminoacylated with tyrosine (Kohl and Hall, 1974), this did not occur with two assayed isolates of TAV (Fernández-Cuartero *et al*., 1994; Joshi and Haenni, 1986).

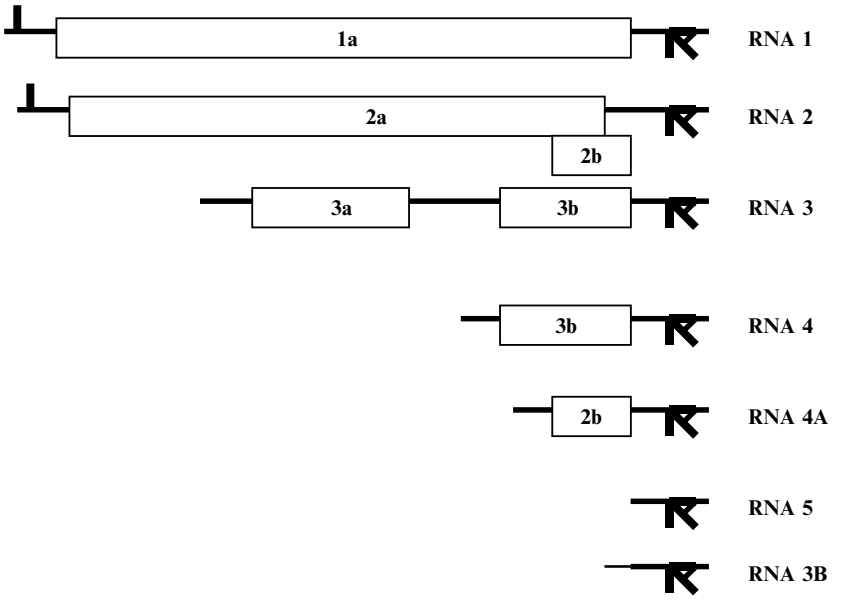

FIG 1. Genome organization of cucumoviruses. The genomes of *Cucumber mosaic virus* (CMV), *Peanut stunt virus* (PSV), and *Tomato aspermy virus* (TAV) each consist of three genomic RNAs (RNAs 1–3) and two major subgenomic RNAs (RNAs 4 and 4A). CMV and TAV also contain a minor RNA (RNA 5), whereas TAV also contains a second minor RNA (RNA 3B). RNA 1 encodes one open reading frame, whereas RNAs 2 and 3 each encode two open reading frames. Expression of the 3′ proximal gene on RNAs 2 and 3 is from the subgenomic RNAs 4A and 4, respectively. RNA 3B is generated from the 3′-noncoding region of TAV RNA 3, which has a duplicated region of 163 nucleotides (the thin line shown at the 5′ end of RNA 3B), whereas RNA 5 is generated from the 3′-noncoding regions of RNAs 2 and 3 of CMV and RNA 3 of TAV (just beyond the duplicated region). Minor RNAs of PSV have not been characterized. Open reading frames in RNAs 1, 2, and 3 are designated 1a, 2a, 2b, 3a, and 3b. The specific locations and sizes of these open reading frames are given in Table I.

The complete nucleotide sequences of 13 isolates of CMV, 2 of TAV, and 3 of PSV have been reported in the data banks (see Table I). In addition, partial sequences have been deposited for 32 CMV isolates, 4 TAV isolates, and 1 PSV isolate. The sizes of the genomic RNAs vary for the different isolates and species. Table I gives the sizes of RNAs 1, 2, and 3 of the isolates for which complete sequences have been reported. RNA 1 is monocistronic, encoding protein 1a in a single open reading frame (Fig. 1). RNA 2 is bicistronic, encoding proteins 2a and 2b in two different reading frames (2b is +1 with respect to 2a), which overlap in part (Fig. 1). RNA3 is also bicistronic, encoding

TABLE I
SIZE OF GENOMIC RNAS AND POSITIONS FOR OPEN READING FRAMES (ORF) IN THE CUCUMOVIRUSES[a]

	CMV-I	CMV-II	TAV	PSV
RNA 1	**3357–3365**	**3389–3391**	**3410–3412**	**3351–3357**
1a ORF	95–98 to 3076–3079	96–98 to 3073–3078	95 to 3076	86–91 to 3094–3109
RNA 2	**3036–3060**	**3038–3053**	**3074**	**2942–2947**
2a ORF	78–97 to 2652–2673	93 to 2612–2615	88 to 2574	77–81 to 2578–2585
2b ORF	2414–2432 to 2746–2836	2409–2413 to 2712–2715	2445–2447 to 2734	2391–2397 to 2675–2682
RNA 3	**2213–2220**	**2197–2209**	**2222–2386**	**2170–2188**
3a ORF	120–123 to 959–973	96–97 to 935–936	89–90 to 931–932	117–120 to 980–989
CP ORF	1255–1263 to 1011–1918	1220–1232 to 1876–1888	1227 to 1883	1243–1249 to 1896–1926

[a] Data are number of nucleotides for RNAs 1, 2, and 3 and nucleotide positions for start and end of the five open reading frames. Data are from CMV isolates Indonesia, Ixora, Tfn, NT9, Legume, Y, Mf, Fny, and SD in subgroup I (CMV-I), CMV isolates Trk7, LS, Ly, and Q in subgroup II (CMV-II), TAV isolates V and KC, and PSV isolates J, ER, and W, for which the complete nucleotide sequence of the three genomic RNAs appears in databases.

protein 3a (the movement protein) and the capsid protein (3b) in two nonoverlapping reading frames separated by a noncoding, intercistronic region of 258–302 nucleotides (nt) (Fig. 1), the length of which depends on the isolate and species. The nucleotide positions of the five open reading frames for the three different species of *Cucumovirus* are indicated in Table I. The open reading frames for proteins 1a, 2a, 3a, and capsid protein are found in all genera of the family *Bromoviridae*, whereas the open reading frame for the 2b protein is only found in the genera *Cucumovirus* and *Ilarvirus*. Although the open reading frame for protein 2b is similarly positioned in these two genera, the protein size and sequence are not conserved between them (Xin *et al*., 1998). Within the genus *Cucumovirus*, the initiation codon for the 2b gene is at a different position for each species. The data strongly suggest that the 2a gene was the ancestral open reading frame and that the 2b gene had arisen *de novo* more than once by overprinting (Roossinck, 2002).

Proteins 1a, 2a, and 3a are translated from genomic RNAs 1, 2, and 3, respectively (Schwinghamer and Symons, 1977). The 2b and capsid proteins are translated from subgenomic messenger RNA 4A and

RNA 4 (Fig. 1), respectively (Schwinghamer and Symons, 1975; Ding *et al*., 1994), which themselves are 3′ coterminal with RNAs 2 and 3, respectively (Ding *et al*., 1994; Gould and Symons, 1978). RNA 4 is 1010–1250 nt, whereas RNA 4A is 630–702 nt (Ding *et al*., 1994; Palukaitis *et al*. 1992). RNA 4 is encapsidated by all three cucumoviruses, whereas RNA 4A is encapsidated by strains of CMV subgroup II and TAV, but either not by strains of CMV subgroup I (Palukaitis *et al*., 1992; Shi *et al*., 1997a) or only at very low levels (Blanchard *et al*., 1997).

In addition to RNAs 1, 2, 3, 4, and 4A, cucumoviruses also encapsidate several other smaller RNAs. RNA 5 of the subgroup II Q strain of CMV is 304–307 nt and is a heterogeneous mixture of the 3′-noncoding region of RNAs 2 and 3 (Fig. 1; Blanchard *et al*., 1996). RNA 5 of the V strain of TAV is 323 nt and is identical to the 3′ end of TAV RNA 3 (Shi *et al*., 1997b). RNA 3B of V-TAV is 486 nt and also derives from the 3′ end of RNA 3, containing a tandem duplication of 163 nt present in TAV RNA 3 (Fig. 1), but not in CMV RNA 3 (Shi *et al*., 1997b). The function of these noncoding, subgenomic RNAs remains a matter of speculation. CMV particles also encapsidate RNA 6, a mixture of plant tRNA and CMV RNA fragments of 70–80 nt (Palukaitis *et al*., 1992).

Defective RNAs derived from RNA 3 by in-frame deletions of ~150–300 nt within the 3a gene have been described in the Fny and M strains of CMV (Canto and Palukaitis, 1998; Graves and Roossinck, 1995). Fny–CMV defective RNAs were supported by other CMV and PSV isolates, were encapsidated, and had no effect on the yield or pathogenesis of the homologous and heterologous helper viruses (Graves and Roossinck, 1995). One defective RNA derived from Fny–CMV RNA 3 was found to be replicated and encapsidated in tobacco (*Nicotiana tabacum*), although this defective RNA was replicated but not encapsidated in zucchini squash (*Cucurbita pepo*) (K.C. Lee, I. Kaplan, S.-M. Wong, and P. Palukaitis, unpublished data).

In any particular isolate, the sequences of the 5′-noncoding region of RNAs 1 and 2 and the 3′-noncoding region of RNAs 1, 2, and 3 are highly conserved. The 5′-noncoding region of all three genomic RNAs has motifs conserved with the internal control regions (ICR) of the promoters of tRNA genes (Marsh *et al*., 1989) that are promoters for (+) strand viral RNA synthesis (Pogue *et al*., 1992). Similar sequences are found in the intergenic region of RNA 3, which also has the promoter sequences for transcription of the subgenomic RNA 4 (Boccard and Baulcombe, 1993). By analogy to the extensively analyzed bromoviruses, the 3′-noncoding region has all the information required for replicase binding and synthesis of the complementary RNA strand (Kao, 2002). Other

functions, perhaps related to translation, must be encoded by the 3′-noncoding region, as clustering of sequences was observed by the particular RNA rather than by strain in a phylogenetic analysis of 15 CMV isolates (Roossinck, 2002). The different levels of RNAs 1, 2, and 3 could also be regulated by the 3′-noncoding region (Duggal *et al*., 1992).

III. Protein Functions

The five proteins encoded by CMV have been analyzed for various functions. Most of this work has been at the genetic level, but some of the studies have been done using purified proteins.

A. 1a Protein

The 1a protein is encoded by RNA 1 (Fig. 1). The 1a protein has been shown to be a component of the isolated CMV replicase (Hayes and Buck, 1990) and has been localized to the vacuolar membrane, the tonoplast (Cillo *et al*., 2002). Sequences conserved in the replication-associated proteins of cucumoviruses and those of other viruses suggest that the 1a protein has two functional domains: an N-terminal proximal domain that is a putative methyltransferase domain involved in the capping of genomic and subgenomic RNAs and a C-terminal proximal domain that is a putative helicase (Gorbalenya *et al*., 1988; Habili and Symons, 1989; Hodgman, 1988; Rozanov *et al*., 1992). These two domains of the CMV 1a protein are known to interact with each other in the yeast two-hybrid system (O'Reilly *et al*., 1998). In addition, the CMV 1a protein interacts with the N-terminal region of the 2a protein in the yeast two-hybrid system (Kim *et al*., 2002). While the C terminus of the CMV 1a protein could be modified by the addition of six histidine residues, the N terminus of the 1a protein lost functionality after such modification (Gal-On *et al*., 2000). However, fusion of the green fluorescent protein (GFP) to the C terminus of the CMV 1a protein prevented virus replication (T. Canto and P. Palukaitis, unpublished data). Sequences involved in eliciting a hypersensitive response in tobacco (Lakshman and Gonsalves, 1985) and sequences important in the seed transmission of CMV (Hampton and Francki, 1992) have been mapped to RNA 1, presumably to the 1a protein. Similarly, CMV RNA 1 was shown to affect the rate of systemic infection in zucchini squash (Gal-On *et al*., 1994). The CMV 1a protein, expressed transgenically in tobacco at subliminal levels, could complement replication and allow systemic movement of CMV RNAs

2 and 3 (Canto and Palukaitis, 1998). Thus, even very low levels of 1a protein are sufficient for replication.

The methyltransferase domain has not been characterized per se in any *Cucumovirus*. In the case of CMV, sequences that affect temperature-sensitive replication of the Sny strain of CMV in muskmelon (*Cucumis melo*) have been mapped to the methyltransferase domain (Bao *et al*., 1999). The 1a protein has been shown to bind *S*-adenosyl methionine, which is required for methylation of the 5′ cap structure (Bao *et al*., 1999). However, the site of *S*-adenosyl methionine binding has not been identified. The methyltransferase domain of the CMV 1a protein is also capable of interacting with itself in the yeast two-hybrid system (O'Reilly *et al*., 1998).

The helicase domain contains a number of motifs characteristic of other helicases (Kadaré and Haenni, 1997). These include an NTP-binding region (motifs I and II) and an ATPase activity and possibly RNA-binding activity (motif VI). Deletions within motifs I, III, and VI of the 1a protein of Fny–CMV inhibited the ability of the 1a protein to function in CMV replication. Moreover, CMV RNA 1 containing any of these deletions could not be replicated in *trans* by 1a protein expressed transgenically in tobacco (T. Canto and P. Palukaitis, unpublished results). Similarly, transgenic tobacco plants expressing CMV RNA 1 containing deletions in helicase motifs I and III were not able to produce a functional 1a protein that could replicate RNAs 2 and 3 (T. Canto and P. Palukaitis, unpublished results). Sequences adjacent to motif VI in the CMV 1a protein were shown to be important for the replication of satellite RNA in zucchini squash (Roossinck *et al*., 1997), which may reflect the putative role of motif VI in unwinding double-stranded RNA. The helicase domain is more resistant to the action of proteinases than the rest of the 1a protein. This was shown for the 1a protein of CMV and two bromoviruses (O'Reilly *et al*., 1995). Although the region of interaction between 1a and 2a proteins has not been mapped in the 1a protein, based on analogous work done with BMV (O'Reilly *et al*., 1995), the C-terminal half of cucumovirus 1a would be expected to be involved in the interactions with the 2a protein.

B. 2a Protein

The 2a protein is encoded by RNA 2 (Fig. 1) and has been localized to the tonoplast (Cillo *et al*., 2002). The 2a protein contains a number of motifs characteristic of RNA-dependent RNA polymerases (Bruenn, 1991; Habili and Symons, 1989; Poch *et al*., 1989). While none of these sequences have been mutated in any cucumovirus to demonstrate

their function in virus replication, such experiments have been done for the 2a protein encoded by the *Alfamovirus Alfalfa mosaic virus* (van Rossum *et al*., 1996). CMV 2a sequences within and adjacent to polymerase motif D (Poch *et al*., 1989) have been identified as affecting a hypersensitive response and systemic infection in cowpea (*Vigna unguiculata*) (Kim and Palukaitis, 1997). Moreover, these sequences, plus an additional one in polymerase motif B, affected systemic infection in bean (*Phaseolus vulgaris*) and pea (*Pisum savitum*) (Kim and Palukaitis, 1993). It was suggested that the former sequence changes might alter a phosphorylation site in the 2a protein (Kim and Palukaitis, 1997). The CMV 2a protein has been shown to be phosphorylated in at least three domains, one of which includes the domain encompassing the sequence changes (Kim *et al*., 2002). However, the sites of phosphorylation in the 2a protein have not been localized. The CMV 2a protein has been shown to interact with the 1a protein in the yeast two-hybrid system, as well as *in vitro*. Only the N-terminal 126 amino acids of the CMV 2a protein were required for this interaction between 1a and 2a proteins, both *in vivo* and *in vitro* (Kim *et al*., 2002). In addition, phosphorylation *in vitro* of this domain was sufficient to block the interaction between 1a and 2a proteins (Kim *et al*., 2002). This indicates that phosphorylation may be a regulator of the formation of the replicase complex. However, phosphorylation was observed only late during infection, suggesting that it results in a pool of 2a protein that is not involved in replication, but rather may be involved in some other function associated with the 2a protein. Such a function might be virus movement, as failure of Fny–CMV to infect pea plants was not associated with inhibition of replication in pea protoplasts (J.-H. Kim and P. Palukaitis, unpublished data).

Some transgenic tobacco plants expressing the 2a protein of CMV showed resistance to CMV infection (Wintermantel and Zaitlin, 2000). Complementation of the replication of CMV RNAs 1 and 3 could be observed in transgenic tobacco lines that did not show resistance to infection by CMV. However, the level of replication was very low, indicating that transgenic expression of the 2a protein was much more limiting for replication than transgenic expression of the 1a protein (T. Canto and P. Palukaitis, unpublished results).

C. 2b Protein

The 2b protein is also encoded by RNA 2, but is expressed from a subgenomic RNA designated RNA 4A (Fig. 1) (Ding *et al*., 1994). RNA 4A was so designated because it appeared as a shoulder on the peak of

RNA 4 in fractionated sucrose gradients (Peden and Symons, 1973). The 2b protein of CMV and TAV has been shown to be a suppressor of posttranscriptional gene silencing (Brigneti *et al*., 1998; Li *et al*., 1999). Specifically, the CMV 2b protein has been shown to inhibit the ability of the gene silencing signal to activate gene silencing in distant tissues, as well as to interfere with DNA methylation in such tissues (Brigneti *et al*., 1998; Guo and Ding, 2002). It has been suggested that the 2b protein may inactivate the mobile signal inducing systemic silencing (Guo and Ding, 2002). Presumably, nuclear localization of the 2b protein (Lucy *et al*., 2000; Mayers *et al*., 2000) facilitated its ability to inhibit DNA methylation, since mutations in the 2b protein that interfered with nuclear localization interfered with the post-transcriptional gene silencing function (Lucy *et al*., 2000). The 2b protein of both CMV and TAV is a single-stranded RNA binding protein (S. W. Ding, personal communication) and also functions as a transcriptional activator in yeast (Ham *et al*., 1999). Deletion of the C-terminal 15–16 amino acids of the CMV 2b protein inhibited this transcriptional activation (Ham *et al*., 1999), virus accumulation in systemic leaves, and virulence expression (Ding *et al*., 1995b), but not nuclear localization or silencing suppression (Lucy *et al*., 2000). Thus, virulence determination and silencing suppression are not necessarily linked properties of the 2b protein. Expression of the 2b protein from either TMV or *Potato virus X* (PVX) viral vectors enhanced the virulence of those viruses, indicating that the 2b protein might be a factor in CMV-mediated synergy (Brigneti *et al*., 1998; Li *et al*., 1999). This suggests that the 2b protein may inhibit host responses interfering with virus infection; in fact, the CMV 2b protein was shown to inhibit salicylic acid-mediated virus resistance (Ji and Ding, 2001). It has been proposed that this effect might be due to salicylic acid potentiating an RNA-mediated antiviral defense that is targeted by the 2b protein.

It appears that the 2b protein also has some direct role in virus movement. Hybrid viruses containing substitution of the (subgroup II) Q–CMV 2b gene by the 2b gene of either the more virulent (subgroup IA) WAII–CMV or V-TAV led to hypervirulence, but not to more rapid accumulation in systemic leaves (Shi *et al*., 2002). Thus, hypervirulence associated with the 2b protein is not determined by differences in the rate of movement. However, deletion of the 2b gene from CMV either prevented or reduced the rate of systemic infection, depending on the host species (Ding *et al*., 1995b; Soards *et al*., 2002) and the age of the plant (Ji and Ding, 2001). In addition, the 2b gene was found to be essential for the movement of pseudorecombinant

viruses formed between CMV RNAs 1 plus 2 and TAV RNA 3 (Shi *et al*., 2003). The N-terminal seven amino acids of the 2b protein encoded by Fny–CMV were not required for promoting systemic infection in a range of CMV hosts, but did have a strong effect on the level of virulence and the level of virus accumulation, with both reduced in all hosts tested (I. Kaplan and P. Palukaitis, unpublished results). In tobacco, there was also an effect observed on the types of cells infected by CMV without a 2b gene. CMV without a 2b gene showed a reduced spread through epidermal cells and an increased spread through mesophyll cells, vis-à-vis the spread pattern of the wild-type virus (Soards *et al*., 2002). However, it is unclear whether these effects were due to a specific effect on promoting virus movement directly or due to the loss of the antiviral defense function of the 2b protein, inhibiting host responses restricting viral movement.

The 2b protein was also shown to promote the selection of recombinant viruses involving the 3′-nontranslated region of TAV RNA 3 (B. Shi, R. H. Symons, and P. Palukaitis, unpublished results). While this may suggest some role for the 2b protein in replication, the 2b protein was not found associated with cellular components containing replication complexes (Cillo *et al*., 2002; Mayers *et al*., 2000). Thus, the role of the 2b protein in recombination may be indirect, by selecting for which sequence combinations of recombinant and pseudorecombinant viral RNA are mobilized (Shi *et al*., 2003).

A tobacco protein (designated 2bip) has been found to interact with the 2b protein, both in the yeast two-hybrid system and *in vitro* (Ham *et al*., 1999). 2bip was expressed constitutively and was found in all plant organs examined. The 2bip showed sequence similarity to an unknown protein from *Arabidopsis thaliana* and to LytB of the cyanobacterium *Synechocystis* sp. (Ham *et al*., 1999). The latter protein is probably involved in regulating the expression of another bacterial protein, which suggests that 2bip may be a regulatory protein. The interaction between the 2b protein and 2bip also occurred for a 2b mutant with a deletion of the C-terminal 15 amino acids (Ham *et al*., 1999), suggesting that this interaction is not associated with the hypervirulence function of the 2b protein.

D. 3a Protein

The 3a protein is encoded by RNA 3 (Fig. 1). There is no requirement for the 3a protein in virus replication, but this protein is essential for virus movement (Boccard and Baulcombe, 1993; Canto *et al*.,

1997; Suzuki *et al*., 1991). The 3a protein is a single-stranded, nucleic acid-binding protein, with no sequence specificity (Li and Palukaitis, 1996). The 3a protein can bind GTP and, to a lesser extent, UTP *in vitro* (Li and Palukaitis, 1996) and is phosphorylated *in vivo* in transgenic plants (Matsushita *et al*., 2002). Movement mediated by the 3a protein could be complemented in *trans*, although with a brief delay, by expression of the 3a protein in transgenic tobacco plants (Kaplan *et al*., 1995; Sanz *et al*., 2000). The role of phosphorylation of the 3a protein in virus movement has not been determined. The 3a protein has been localized to plasmodesmata between infected cells (Blackman *et al*., 1998; Vaquero *et al*., 1996), as well as to large aggregates inside sieve elements (Blackman *et al*., 1998). In microinjection experiments, the 3a protein could gate the movement through plasmodesmata of itself, RNA (Canto *et al*., 1997; Ding *et al*., 1995a), or fluorescent dyes (Canto *et al*., 1997; Ding *et al*., 1995a; Vaquero *et al*., 1994). Mutation of amino acids Tyr-144/Asp-145 to alanine inhibited these gating functions as well as viral movement in plants (Ding *et al*., 1995a; Li *et al*., 2001). Various other mutations also inhibited the movement functions of the 3a protein (Li *et al*., 2001; Nagano *et al*., 2001). The RNA-binding domain has been identified by deletion analysis in two separate studies and involves either amino acids 174–233 (Vaquero *et al*., 1997) or amino acids 118–160 (You *et al*., 1999). The actual site of RNA binding after UV cross-linking to the native 3a protein has not been determined. Mutational analyses also showed that some sequences mediate interaction with plants in a species-specific manner. Thus, mutations of amino acids 51 and 240 of the 3a protein increased the level of 3a protein accumulation in tobacco up to 50-fold (Gal-On *et al*., 1996), and increased the efficiency of systemic movement (Gal-On *et al*., 1995), but inhibited the ability of the same virus to move systemically in cucurbit species (Kaplan *et al*., 1997). Similarly, mutation of amino acids Asp-20/Asp-21 to alanine prevented local lesion formation in *Vigna unguiculata* and *Chenopodium quinoa* (Li *et al*., 2001). In *Chenopodium amaranticolor*, this phenotype was shown to be due to the inhibition of cell-to-cell movement by this mutation (Canto and Palukaitis, 1999a).

The 3a protein was able to form tubules on the surface of infected tobacco protoplasts (Canto and Palukaitis, 1999b). No such tubules were seen penetrating plasmodesmata in infected *Nicotiana clevelandii* plants (Blackman *et al*., 1998), and while mutation of amino acids 20/21 inhibited tubule formation, there was no effect on systemic infection in a number of host species examined (Li *et al*., 2001). However, this

mutation did affect movement between epidermal cells in tobacco and also prevented local lesion formation in several plant species (Canto and Palukaitis, 1999a, 1999b; Li *et al*., 2001).

The 3a protein is also required for long-distance movement. Mutation of amino acid Pro-60 to alanine did not affect cell-to-cell movement, but had a temperature-sensitive effect on long-distance movement (Li *et al*., 2001). The various mutants affecting cell-to-cell movement (Li *et al*., 2001) could not complement the mutant affecting long-distance movement, suggesting that the same 3a protein molecules involved in one activity must also be involved in other activities required for movement. Moreover, some of these mutations also inhibited RNA binding (Li, 1995) as well as plasmodesmal localization (T. Canto and P. Palukaitis, unpublished results).

E. Capsid Protein

The 3b or capsid protein is encoded by RNA 3 (Habili and Francki, 1974c), but is expressed from the subgenomic RNA 4 (Fig. 1) (Schwinghamer and Symons, 1975). The capsid protein is the sole protein associated with virus particles. CMV and TAV particles have been crystallized and their three-dimensional structures have been resolved. The analysis of the virus structures was described (see Section II,B).

The capsid protein is also required for virus movement, within and between plants (Boccard and Baulcombe, 1993; Canto *et al*., 1997; Mossop and Francki, 1977; Suzuki *et al*., 1991). The CMV capsid protein has been shown to be required for cell-to-cell movement, although this appears to be in an indirect role (see Section IV,A). However, there are also determinants in the capsid protein that affect cell-to-cell movement in some hosts (Kaplan *et al*., 1998; Ryu *et al*., 1998a; Wong *et al*., 1999), suggesting some interaction with the host and not just with other viral components in promoting cell-to-cell movement. The capsid protein is clearly involved in long-distance movement (see Section V,B), and it is unlikely that this function merely relates to its ability to protect viral RNAs from degradation. There are determinants associated with the capsid protein affecting long-distance movement in several hosts (Ryu *et al*., 1998a; Saitoh *et al*., 1999; Takeshita *et al*., 2001; Taliansky and García-Arenal, 1995; Wong *et al*., 1999). The capsid protein also contains determinants for the transmission of the virus from plant to plant via the aphid vectors of cucumoviruses (see Section V,C).

IV. Replication and Gene Expression

A. Replicase Composition, Architecture, and Localization

The CMV replicase is membrane bound (Jaspars *et al*., 1985) and contains viral-encoded as well as host-encoded components (Hayes and Buck, 1990). There are at least three components, and probably more. These include the CMV 1a and 2a proteins, as well as a host protein of 50 kDa (Hayes and Buck, 1990). In addition, proteins of 100, kDa 50, and 32 kDa were found to bind to the 3′-terminal sequences of (−) stand RNA of CMV (Hayes *et al*., 1994a). The roles of the various host proteins in the replication process are unknown. They may be functional proteins that participate in the synthesis of the viral RNAs or capping reactions, or they may be structural proteins, providing sites of anchorage of the replicase to host membranes. Neither the molecular size nor the structure of the CMV replicase has been determined. The CMV replicase is associated with vacuolar membranes, as both 1a and 2a proteins along with low levels of (−) CMV RNAs have been localized to electron-dense structures associated with this membrane (Cillo *et al*., 2002). Fractionation studies showed that the CMV 2a protein was detected in both a soluble and membrane-bound form, while the 1a protein was detected only in a membrane-bound form (Gal-On *et al*., 2000). By analogy to BMV (Chen and Ahlquist, 2000; den Boon *et al*., 2001) and the similarities in structure between BMV and CMV 1a proteins (O'Reilly *et al*., 1998), it seems likely that the CMV 1a protein either is associated directly with the tonoplast or anchors to a tonoplast membrane protein. Presumably, the 2a protein then becomes associated with the tonoplast by its association with the 1a protein. Other host proteins may then become associated with either 1a or 2a proteins, as well as with the various host proteins binding to any of the above proteins. Later in infection, newly synthesized 2a protein is phosphorylated, preventing its interaction with the anchored 1a protein (Kim *et al*., 2002). This probably leads to the accumulation of free, soluble 2a protein, which presumably is no longer involved in replication, but instead may be involved in some other function such as movement (see Section III,B).

Vesicles associated with tonoplast membranes were observed previously in plants infected by CMV and TAV (Hatta and Francki, 1981). These were suggested as being involved in replication, as they contained dense bodies that appeared to be double-stranded RNA (Hatta and Francki, 1981). However, these vesicles were observed in older

infected tissues, and their role in replication is not clear, as replication is an early function.

In the yeast two-hybrid system, it has been shown that the CMV 1a protein interacts with itself in two ways (O'Reilly *et al*., 1998). That is, the N-terminal half of the 1a protein interacts with the same region of a second molecule of the 1a protein, as well as with the C-terminal half of the 1a protein (O'Reilly *et al*., 1998). It has been suggested that the latter reaction is intramolecular, preventing the interaction of the C-terminal half of the 1a protein with the N-terminal region of the 2a protein until the N-terminal regions of different molecules of 1a protein interact with each other. This would free the C-terminal regions of each 1a protein to interact with 2a protein molecules (O'Reilly *et al*., 1998). A domain in the 2a protein consisting of some or all of amino acids 1–126 is necessary and sufficient for the interaction with the C-terminal region of the 1a protein (Kim *et al*., 2002). Phosphorylation of this domain, *in vitro*, prevented the 2a protein from interacting with the 1a protein (Kim *et al*., 2002).

The 1a protein also binds *S*-adenosyl methionine (Bao *et al*., 1999), which is required for the methylation of 5′ cap structures added to *de novo*-synthesized RNA. Antibody probing suggests that the methyl transferase domain and the helicase domain of the 1a protein are on the surface of the CMV replicase, as is the GDD motif of the 2a protein. Interactions of these domains with monoclonal antibodies led to inhibition of enzyme activity (Hayes *et al*., 1994b). These are domains expected to be accessible to RNA templates and the substrates required for RNA synthesis and RNA capping.

B. Enzymology

Little is known about the requirements for cucumovirus replication. Largely, this results from the complicated history of the purification of RNA-dependent RNA polymerases (RdRp), as most of the factors required for RNA synthesis were analyzed using partially purified polymerases in the 1970s and 1980s. The initial strategy for RdRp purification showed there was little to no RdRp present in noninoculated healthy plants, and so it was assumed that all RdRp activity would be due to a viral-specific polymerase. However, this strategy proved incorrect, as host-encoded RdRp were found to be stimulated by stress, particularly by virus infection (reviewed by Fraenkel-Conrat, 1986). A host RdRp was purified and characterized from tomato plants (*Lycopersicon esculentum*) in the 1990s (Schiebel *et al*., 1993a, 1993b), and the gene encoding this 127-kDa protein was cloned and

characterized several years later (Schiebel *et al*., 1998). Thus, during the 1970s and 1980s, the enzymological characterization of a number of so-called viral replicases was in fact done on either the host RdRp or a mixture of the host RdRp and the viral replicase. This was further complicated by observations made in the late 1970s and early 1980s that there were two types of host RdRp: a soluble RdRp and a membrane-associated RdRp. In the case of CMV, both of these host-encoded activities were purified and characterized separately (Gill *et al*., 1981; Kumarasamy and Symons, 1979). Moreover, both the soluble enzyme (Kumarasamy and Symons, 1979) and the solubilized, membrane-associated enzyme (Gordon *et al*., 1982) were shown to be devoid of CMV-encoded proteins. Subsequent purification was based not only on measuring the enzyme activity, but also by analyzing the products obtained; the host RdRp synthesized predominantly small RNAs, whereas the viral-encoded RdRp completed the synthesis of double-stranded RNA molecules *in situ* (Jaspars *et al*., 1985). This approach facilitated the purification of the viral-encoded RdRp. In one case, this yielded an activity that was stable, after about 9000-fold purification, but only was able to synthesize (−) RNA complementary to the added (+) RNA (Quadt and Jaspars, 1991). A different purification protocol yielded a true replicase in that it was able to synthesize double-stranded RNAs as well as nascent (+) single-stranded RNAs from added (+) single-stranded CMV RNAs after purification approaching 1,000,000-fold (Hayes and Buck, 1990). However, the purified enzyme was unstable. Nevertheless, both of these enzyme preparations could synthesize (+) and (−) RNA from added CMV satellite RNA (Hayes *et al*., 1992; Wu *et al*., 1993). In the absence of a stable, homogeneous replicase preparation, the K_d and K_m for templates and substrates have not been ascertained. More recently, other approaches have been used to obtain CMV RdRp preparations that, while not highly purified, were template dependent and could be used for *in vitro* assays (Gal-On *et al*., 2000; Sivakumaran *et al*., 2000). One of these preparations has been used to localize RNA sequences important for replication and subgenomic RNA production (Chen *et al*., 2000; Sivakumaran *et al*., 2000, 2002).

C. Replication and Subgenomic Promoters

Sequences in CMV RNA 3 have been identified that are essential for replication *in vivo* (Boccard and Baulcombe, 1993). Retaining the first 92 nt of the 95 nt 5′-nontranslated region gave near wild-type accumulation of RNA 3, while retaining the first 64 nt gave levels of

accumulation of near 15% that of wild-type RNA 3. This was reduced to less than 1% of wild-type RNA 3 accumulation when the 5′-nontranslated region was only 47 nt (Boccard and Baulcombe, 1993). Interestingly, Fny-CMV with a deletion of nucleotides 746 to 947 in the 3a gene and passaged on 3a-transgenic tobacco generated a deletion in RNA 3 extending from nucleotides 91/92 in the 5′-nontranslated region to nucleotide 1033/1034 in the intergenic region (Kaplan and Palukaitis, 1998). This confirms that sequences 5′ of nucleotide 91/92 are essential for maximum accumulation of RNA 3. However, the first 4 nt of the 5′-nontranslated region (GUUU) are not essential, but rather are preferred for replication of TAV RNAs 1 or 2. In addition, three U residues can be added within this region with no obvious effects on TAV replication (B. J. Shi, P. Palukaitis, and R. H. Symons, unpublished results). Sequences in the open reading frame for 3a protein also have an effect on RNA 3 accumulation. The change C100A in V-TAV RNA 3 resulted in an eightfold increase in its accumulation (Moreno *et al*., 1997b).

The intergenic region was shown to be necessary for CMV RNA 3 accumulation (Boccard and Baulcombe, 1993). However, the aforementioned spontaneous deletion in CMV RNA 3 from nucleotides 91/92 to 1033/1034 extended 77 nt into the intergenic region (Kaplan and Palukaitis, 1998), indicating that some of this region was dispensable for RNA 3 accumulation. The reason for the requirement of the intergenic region for replication of RNA 3 is not certain, but may relate to the presence of ICR motifs related to the ICR promoters of eukaryotic tRNAs (Marsh and Hall, 1987; Marsh *et al*., 1989; Pogue *et al*., 1992). The aforementioned defective RNA 3 contained a deletion from within one ICR-1-like motif in the 5′-nontranslated region extending to another ICR-1-like motif within the intergenic region (Kaplan and Palukaitis, 1998). This suggests that both of these ICR-1-like motifs may not be necessary for replication, in contrast to conclusions from earlier deletion analyses (Boccard and Baulcombe, 1993). However, the differences in observations could also relate to spatial differences between the remaining ICR-like elements and other promoter-like elements in the RNA 3 molecule (Marsh and Hall, 1987; Marsh *et al*., 1989; Pogue *et al*., 1992).

Promoter sequences in the 3′-terminal tRNA-like structure of RNA 3 have been identified that are required for the synthesis of (−) CMV RNA by the CMV replicase (Boccard and Baulcombe, 1993; Sivakumaran *et al*., 2000). *In vitro*, (−) RNA synthesis initiates from the penultimate C residue of the 3′-terminal CCA sequence, using either a template corresponding to the 3′ 208 nt of Fny–CMV RNA 3 or a core stem–loop structure corresponding to stem–loop C of the 3′-terminal tRNA-like

structure (designated as various SLC derivatives). SLC-like elements consisting of 36 nt and containing the 3′-terminal 6 nt were shown to be sufficient for replicase binding and initiation of (−) RNA synthesis (Sivakumaran *et al*., 2000). Mutations made in the loop at the end of the stem, as were used to demonstrate that an invariant 5′-CA-3′ motif (either as part of the trinucleotide loop and a flanking base pair or as part of a pentanucleotide loop, depending on the CMV strain and particular RNA), were required for replicase interaction (Sivakumaran *et al*., 2000).

The core promoter for *in vitro* (+) RNA synthesis by the CMV replicase differs in CMV RNAs 1 and 2 vs RNA 3. Near the 3′ terminus of the (−) strand of the CMV RNAs are conserved elements, consisting either of ICR-2-like sequences (for RNAs 1 and 2) or a repeating 5′-CA-3′ dinucleotide (for RNA 3). These are required for (+) RNA synthesis from a (−) RNA template. A template consisting of the 3′ 26–nt residues of (−) CMV RNA 3 and containing an extra G residue at the 3′ terminus was sufficient to promote (+) RNA synthesis *in vitro*, initiating from the penultimate C residue (Sivakumaran *et al*., 2000). The 3′-terminal G and penultimate C residue were essential for the initiation of RNA synthesis (Sivakumaran *et al*., 2000). A different model for promotion of (+) RNA synthesis *in vivo* for BMV and CMV RNAs 1 and 2 was suggested. This was based on the mutation of nucleotides present in the stem–loop structure of (+) BMV RNAs 1 and 2, most of which corresponded to the ICR-2-like element, and examination of the replication of such mutants in protoplasts (Pogue and Hall, 1992). This model stated that maintenance of the stem–loop structure in the 5′ end of the (+) RNA was more important than the maintenance of a similar structure in the 3′ end of the (−) RNA (Pogue and Hall, 1992). The two models are not necessarily inconsistent, since they both demonstrate the necessity for the ICR-2-like element, although they differ in some other details.

Sequences necessary for promoting the synthesis *in vitro* of subgenomic RNA 4 and 4A have been defined (Chen *et al*., 2000; Sivakumaran *et al*., 2002). The initiation site for RNA 4 synthesis begins at sequences complementary to nucleotide 1183 of Fny–CMV RNA 3, in the intergenic region, at an essential C nucleotide (Sivakumaran *et al*., 2000). This is located 5–7 nt 5′ of a stem–loop structure present in all CMV strains (Chen *et al*., 2000). The minimum promoter sequences extend from −28 to +15, relative to the C nucleotide initiation site (Chen *et al*., 2000). These observations are consistent with earlier analyses done *in vivo*, which showed that the core promoter region on (−) CMV RNA 3 for (+) RNA 4 synthesis was between −37 and +40

relative to the initiation site (Boccard and Baulcombe, 1993). A similar organization of the promoter for subgenomic RNA 4A was identified at nucleotides −31 to +13 relative to the initiation site, with three conserved elements: a C nucleotide at the initiation site, in an unpaired sequence complementary to nucleotide 2359 of Fny–CMV RNA 2; a stem–loop structure 3′ of the initiation site extending from nucleotide −4/−6 to −29/−31; and a template rich in A and U residues (Sivakumaran *et al*., 2002).

CMV RNA 5 is contiguous with the 3′ 304–307 nt of CMV RNAs 2 and or 3 (Blanchard *et al*., 1996, 1997; Palukaitis *et al*., 1992). It has yet to be resolved whether RNA 5 is a specific breakdown product of (+) RNA 2/3 or whether it is synthesized via a cryptic subgenomic RNA promoter present in (−) RNAs 2/3. The presence of RNA 3B, contiguous with the 3′ 486 nt of TAV RNA 3 along with an RNA 5, contiguous with the 3′ 323 nt of TAV RNA 3, would suggest that if such RNAs were synthesized via a subgenomic promoter, then the promoter should be present within the common 5′-terminal sequences of RNAs 3B and 5. As has been pointed out (Suzuki *et al*., 2003), the sequence of 20 nt adjacent to the 5′ termini of CMV RNA 5 and TAV RNAs 5 and 3B is almost identical for each of these RNAs as well as with the sequence at the 5′ end of RNA 5 of the unrelated *Beet necrotic yellow vein virus*. In the latter case, this sequence has been identified as part of an internal subgenomic promoter (Balmori *et al*., 1993). Alternatively, the subgenomic promoter for both TAV RNA 3B and RNA 4 might be upstream of the 5′ end of RNA 3B. In that case, because the 5′ 163 nt of RNA 3B is a direct repeat (with one nucleotide difference) of the 5′ 163 nt of RNA 5 (Shi *et al*., 1997b), RNA 5 could be formed by intramolecular, homologous recombination during the synthesis of RNA 3B.

V. Virus Movement

Although many aspects of the basis of movement have been ascertained, there are many complexities still to unravel. These include what host factors are involved in cell-to-cell and long-distance movement, as well as the nature of the barriers to these processes. There are also a number of inconsistencies that have been observed in studies on cucumovirus movement, which will be mentioned, as they exemplify the complexity of the processes involved in virus movement.

A. Cell-to-Cell Movement

Although all CMV-encoded proteins have been shown to have some role in affecting movement, the 3a protein is considered the major movement protein. As this protein supports the movement of all three genomic RNAs, it is not unexpected that the 3a protein is able to function in *trans*. Thus, infection of transgenic tobacco plants expressing the CMV 3a gene, by CMV containing a deleted 3a gene, allowed complementation of movement to occur, resulting in cell-to-cell and systemic movement (Kaplan *et al*., 1995; Sanz *et al*., 2000). Moreover, transgenic expression of the 3a protein also enhanced the rate of movement of wild-type CMV (Sanz *et al*., 2000). The CMV 3a protein is also known to bind to single-stranded RNAs cooperatively (Li and Palukaitis, 1996). The 3a protein binds to the viral RNA and forms a complex that is believed to facilitate movement. This complex probably interacts with host proteins of the plasmodesmata, as CMV 3a protein has been localized to the plasmodesmata (Blackman *et al*., 1998; Vaquero *et al*., 1996). This complex also either transports the RNA through the plasmodesmata or facilitates the movement of RNA, but does not enter plasmodesmata as the elongated complex visualized *in vitro* (Nurkiyanova *et al*., 2001). CMV 3a mutants that affected the extent of cooperative binding did not affect virus movement, as long as the 3a protein was still able to bind RNA efficiently (Li, 1995). In addition, a mutant 3a protein that showed a reduced efficiency of RNA binding, but still bound RNA cooperatively, was able to traffic RNA from cell to cell (Li, 1995). Thus, RNA binding of one form or the other appeared to be essential to promote viral movement. Nevertheless, the 3a protein was able to facilitate its own rapid movement from cell to cell (Ding *et al*., 1995a) and was detected several cells ahead of those accumulating virus in inoculated squash (*Cucurbita pepo*) cotyledons (Havelda and Maule, 2000).

The CMV 3a protein could also generate tubules on the surface of protoplasts (Canto and Palukaitis, 1999b). This suggested that there might be an alternative pathway to movement from that described earlier. However, such tubules were not seen between cells in CMV-infected *Nicotiana clevelandii* (Blackman *et al*., 1998), in contrast to infection by *Olive latent 2 virus* (Grieco *et al*., 1999), a distantly related member of the *Bromoviridae*. Moreover, one of the mutant CMV 3 proteins was unable to form such tubules on the surface of protoplasts, but was able to support both local and systemic movement of CMV in a number of CMV hosts (Canto and Palukaitis, 1999b; Li *et al*., 2001). This suggests that either such tubules are not required for movement

per se or that they are required for movement between particular tissues or in certain host species. However, no such tubules have been observed in *C. pepo* plants, which contain a more complex vein structure, or in *Zinnia elegans*, which is an apoplastic loader of sugar and contains much fewer plasmodesmata between companion cells and sieve elements (J. Forrest and P. Palukaitis, unpublished results).

A number of 3a mutations were made to examine their effects on CMV movement (Li *et al*., 2001). Amino acid changes within the central region of the 3a protein showed a very dramatic effect on virus cell-to-cell movement, although three movement-defective mutants could be complemented for virus movement in tobacco expressing CMV 3a protein (Li *et al*., 2001). One mutant could not be complemented, although it did not affect the accumulation of viral RNAs in single cells (Li *et al*., 2001). While this mutant (with Asn-191/Tyr-192 both changed to alanine) was *cis* dominant for inhibition of the transport of its own RNA, it was not *trans* dominant for interference with the transport of other CMV RNAs. Transgenic *Nicotiana benthamiana* plants expressing any of these movement-defective, mutant 3a proteins did not inhibit the movement of wild-type CMV (F. Cillo and P. Palukaitis, unpublished results). None of the four dysfunctional 3a protein mutants could complement each other for cell-to-cell movement (Li *et al*., 2001). This suggests that 3a protein molecules associated with the viral RNA must be competent for all aspects of the movement process.

Several lines of evidence indicate that both the 3a protein and the capsid protein have a role in cell-to-cell movement. Deletion of either the 3a protein or the capsid protein did not affect the ability of the virus to replicate in protoplasts, but impaired the movement of CMV in plants (Boccard and Baulcombe, 1993; Canto *et al*., 1997; Suzuki *et al*., 1991).

The 28-kDa movement protein of the *Umbravirus Groundnut rosette virus* (GRV) shows strong sequence similarities to the 3a movement protein of CMV (Taliansky *et al*., 1996). Substitution of the CMV 3a gene by the open reading frame encoding the GRV 28-kDa movement protein allowed host-specific movement. The chimeric virus could move cell-to-cell and systemically in *N. benthamiana* and only cell-to-cell in tobacco, but not at all in cucumber (*Cucumis sativus*) (Ryabov *et al*., 1999a). *N. benthamiana* is a host common to CMV and GRV, whereas tobacco and cucumber are hosts of CMV but not of GRV. Thus, there was limited complementation. [GRV does not encode a capsid protein per se, but encodes a 27-kDa movement protein that was shown to facilitate the long-distance movement of TMV (Ryabov *et al*.,

1999b)]. CMV expressing the open reading frame encoding the GRV 28-kDa movement protein in place of its 3a gene, and expressing the gene encoding the GFP in place of its capsid protein gene, was still able to move cell to cell in the aforementioned two *Nicotiana* species, but not long distance in either host (Ryabov *et al*., 1999a). Similarly, replacement of the 3a protein of CMV with the 22-kDa movement protein of the *Tombusvirus Cymbidium ringspot virus* (CymRSV) allowed cell-to-cell movement in hosts common to CymRSV and CMV (Huppert *et al*., 2002). Moreover, neither deletion of the CMV capsid protein gene nor replacement of the capsid protein gene by the gene encoding the GFP in similar constructs prevented cell-to-cell movement by the hybrid virus (Huppert *et al*., 2002). Thus, the capsid protein is required for cell-to-cell movement of CMV, but not for cell-to-cell movement of CMV containing the movement proteins (either the GRV 28-kDa movement protein or the CymRSV 22-kDa movement protein) of viruses that do not require capsid protein for cell-to-cell movement.

The CMV 3a protein and capsid protein could promote the cell-to-cell movement of BMV when both these CMV genes replaced the corresponding genes of BMV (Nagano *et al*., 1999). Hybrid viruses containing heterologous combinations of BMV and CMV capsid proteins and 3a proteins did not undergo cell-to-cell movement, suggesting that these two proteins interact with each other in some specific manner (Nagano *et al*., 1999). When the CMV 3a protein was placed into the genetic background of BMV, it was dysfunctional for movement, even in common hosts. However, spontaneous mutant viruses were obtained from this hybrid virus that did move cell to cell. These mutants had introduced termination codons, resulting in deletion of the C-terminal 33 amino acids of the 3a protein (Nagano *et al*., 1997). A previous study had shown that 43 amino acids could be deleted from the C terminus of the CMV 3a protein and movement was apparently unaffected in tobacco (Kaplan *et al*., 1995), although this observation was not reproducible with a different CMV strain in a more recent study (Nagano *et al*., 2001). Within the genus *Cucumovirus*, cell-to-cell movement also requires specific compatibility of the 3a and capsid protein. Thus a chimeric RNA 3 with the 3a gene of TAV and the capsid protein gene of CMV was unable to move cell to cell in various *Nicotiana* species when coinoculated with CMV RNAs 1 and 2. In contrast, the reciprocal recombinant RNA 3 (CMV 3a gene and TAV capsid protein gene) moved efficiently (Salánki *et al*., 1997). This further supports the need for some interaction by both proteins to facilitate cell-to-cell movement. However, various attempts to observe direct, specific interactions between the capsid protein and the 3a protein have been unsuccessful

(D. Szilassy, T. Canto, and P. Palukaitis, unpublished observations). Moreover, in another apparent contradiction, microinjection of the 3a protein into leaf cells allowed plasmodesmal gating of larger molecules to occur in the absence of capsid protein (Ding *et al*., 1995a; Nguyen *et al*., 1996). Thus, different assays indicate different requirements for movement and have not shown conclusively how the capsid protein participates in cell-to-cell movement.

A model was proposed in which it was speculated that the role of the capsid protein in cell-to-cell movement was to induce a conformational change in the 3a protein, allowing a change in function of the 3a protein (Ryabov *et al*., 1999a). Thus, the *in vitro*-expressed and refolded 3a protein used for microinjection experiments was proposed to contain a mixture of conformational forms, some capable of capsid protein-independent movement (Ding *et al*., 1995a; Nguyen *et al*., 1996). In addition, the GRV 28-kDa movement protein was proposed not to have such alternative conformational states, allowing cell-to-cell movement without capsid protein (Ryabov *et al*., 1999a). In support of this model, CMV expressing the 3a protein containing a deletion of the C-terminal 33 amino acids was shown to be able to move cell to cell in the absence of the CMV capsid protein (Nagano *et al*., 2001). However, such a construct was not able to move long distance, confirming a separate role for capsid protein in cell-to-cell vs long-distance movement. Thus, the picture that emerges at present is that the 3a protein is required for cell-to-cell movement, whereas the capsid protein is not required directly for cell-to-cell movement, but affects cell-to-cell movement in some indirect capacity.

The level of the 3a protein has some effect on the rate of virus movement. CMV showed accelerated movement in transgenic tobacco plants expressing the wild-type movement protein of either CMV (Sanz *et al*., 2000) or TMV (Cooper *et al*., 1995). In contrast, CMV with the 3a gene deleted and inoculated to transgenic tobacco plants expressing the CMV 3a gene exhibited a delay in CMV movement, which also affected the development of symptoms (Kaplan and Palukaitis, 1998; Kaplan *et al*., 1995; Sanz *et al*., 2000). Moreover, TAV expressing the functional 3a protein from only a low proportion of a pool of RNA 3 also exhibited slower movement and less virulence (Moreno *et al*., 1997a). However, the decreased rates of movement did not affect the ultimate accumulation levels achieved by either virus.

Substitution of the genes encoding either the 3a protein or the capsid protein in RNA 3 by the gene encoding the GFP allowed the cells accumulating virus to be visualized. Viruses with such gene replacements showed single cell infections (Canto *et al*., 1997). Coinfection of plant

cells by viral RNAs expressing the GFP in place of the 3a protein or the capsid protein allowed complementation to occur and cell-to-cell movement to be visualized (Canto *et al*., 1997). Curiously, movement of CMV containing the GFP gene replacing the 3a gene did not occur in 3a transgenic tobacco plants. This was not due to the need for particular 3a gene sequences being required for RNA 3 movement in *cis*, as various 3a deletion mutants (including one in which the entire 3a gene plus adjacent, flanking, nontranslated regions have been removed) still showed complementation of movement in 3a transgenic plants (Kaplan and Palukaitis, 1998). Moreover, complementation of the movement of RNA 3 molecules expressing GFP in place of the 3a protein occurred from a coreplicating CMV RNA 3 expressing the wild-type 3a protein as well as GFP in place of its capsid protein (Canto *et al*., 1997). For the same reason, it is also unlikely that this movement dysfunction of CMV containing the gene encoding GFP in place of the 3a gene was due to any inhibitory function by the GFP on CMV movement. Thus, failure of the transgenically expressed 3a protein to complement the movement of CMV expressing GFP in place of the 3a protein remains unexplained.

The early stages of CMV cell-to-cell movement have not been studied in detail. It is known that temperature affects the rate of movement from mechanically inoculated epidermal cells to the mesophyll and that under optimal conditions in cowpea, CMV first moves from the inoculated epidermis to the underlying mesophyll layer in 2–5 hours (Welkie and Pound, 1958). In tobacco, movement from epidermal cells to either other epidermal cells or mesophyll cells is affected by both the 2b protein and the 3a protein (Canto and Palukaitis, 1999b; Soards *et al*., 2002).

B. Long-Distance Movement

The systemic movement of CMV has been shown to follow the path of photoassimilates (Thompson and García-Arenal, 1998) and did not occur in cucumber plants above internodes in which living cells had been steam killed (I. Moreno, J. R. Thompson, and F. García-Arenal, unpublished data.). Hence, it appears that the systemic movement of CMV occurs only through the phloem. It is assumed that CMV RNA moves long distances in the form of virions. This is supported by experiments showing that CMV RNA present in phloem exudate from infected cucumber plants sedimented in sucrose gradients to a similar position as to CMV RNA present in purified CMV particles mixed with phloem exudate from noninoculated plants (A. Requena, I. Moreno,

and F. García-Arenal, unpublished results). However, virions were not seen inside plasmodesmata (Ding *et al*., 1995a), even those between companion cells and sieve elements, although these contained abundant 3a protein (Blackman *et al*., 1998). Because neither translation nor replication can occur in mature sieve elements, it was proposed that RNA and capsid protein would have to be transported (by 3a protein) into sieve elements and assembled there into virus particles (Blackman *et al*., 1998). These particles would then be translocated throughout the vascular system. Deletions of the capsid protein sequences proximal to the N-terminal basic arm had an effect on virus movement independent of effects on encapsidation, again in a host-specific manner (Kaplan *et al*., 1998; Schmitz and Rao, 1998). These data indicated that the capsid protein itself, but not necessarily virions, was essential for long-distance movement and confirmed other conclusions that virion formation was not necessary for cell-to-cell movement, in some hosts.

The cucumoviral capsid protein has host-specific determinants for systemic movement. Strain 1 of TAV was able to infect directly inoculated cotyledons or leaves of cucumber plants similarly to Fny–CMV, but was completely unable to spread systemically. The defective long-distance movement of TAV was complemented by the capsid protein of CMV (Taliansky and García-Arenal, 1995). Similarly, when the capsid protein and the 3′-noncoding region of R–CMV were substituted by the corresponding sequences from P-TAV, the recombinant virus was unable to infect cucumber plants systemically (Salánki *et al*., 1997). Because both CMV and TAV can invade tobacco systemically, these results illustrate the host-specific role of the capsid protein in systemic transport. The defective long-distance movement of 1-TAV in cucumber plants was due to its inability to access the phloem at the minor veins of the inoculated leaves; the bundle sheath–intermediary cell interface was shown to be the boundary to cell-to-cell spread (Thompson and García-Arenal, 1998). Thus, the CMV capsid protein has a role in virus movement by promoting passage through this cellular boundary and accessing the phloem for systemic transport. These data also showed that plasmodesmata between different cell types (i.e., between mesophyll and adjacent bundle sheath cells or between bundle sheath and intermediary cells) are functionally different, with regard to the role of the capsid protein in cell-to-cell movement (Thompson and García-Arenal, 1998).

CMV encoding the CymRSV 22-kDa movement protein in place of the 3a movement protein was able to move long distance in host plant species common to CMV and CymRSV, as well as in some hosts unique

to CMV (Huppert *et al*., 2002). However, if the gene encoding the CMV capsid protein was either deleted or replaced by the gene encoding the GFP in the same CMV/CymRSV hybrid virus, then there was no systemic infection (Huppert *et al*., 2002). Thus, the CMV capsid protein was essential for long-distance movement of these hybrid viruses. Similarly, CMV containing the gene encoding the 28-kDa movement protein of GRV replacing the gene encoding the 3a protein could move cell to cell and long distance in *Nicotiana benthamiana*, a host common to CMV and GRV, but only cell to cell in tobacco, a nonhost for GRV. CMV expressing, the 3a protein containing a deletion of the C-terminal 33 amino acids was shown to be able to move cell to cell in the absence of the CMV capsid protein (Nagano *et al*., 2001). However, such a construct was not able to move long distance, confirming a separate role for capsid protein in cell-to-cell and long-distance movement.

Examination of the barrier to long-distance movement of this hybrid virus in tobacco indicated that the CMV/GRV hybrid virus was unable to either enter or exit sieve elements (Ryabov *et al*., 1999a). However, a variant of the aforementioned hybrid virus, in which the gene encoding the CMV capsid protein was also replaced with the gene encoding the GFP, was unable to infect *Nicotiana benthamiana* systemically. The barrier to systemic infection of this hybrid virus was at the interface between the bundle sheath and the phloem cells (phloem parenchyma and companion cells) (Ryabov *et al*., 1999a). Thus, the 3a protein and capsid protein appear to have different roles in movement between cell types during the systemic infection by CMV.

A CMV 3a protein mutant was described that was temperature sensitive for long-distance movement. This mutant could not be complemented for movement by any of four 3a protein mutants that affected cell-to-cell movement (Li *et al*., 2001). This suggests that the same 3a protein molecule(s) must be able to function in the various roles required for cell-to-cell and long-distance movement.

The capsid protein is required for long-distance movement, but the capsid protein expressed transgenically was not sufficient to overcome this requirement (S. H. Kim, M. Taliansky, and P. Palukaitis, unpublished data). Long-distance movement appeared to be much more efficient in some hosts if the capsid protein could form virions, and long-distance movement required virions in many hosts (Kaplan *et al*., 1998; Suzuki *et al*., 1995). Curiously, long-distance movement did not occur for GFP expressing constructs, even when they expressed both capsid protein and 3a protein, for reasons that are not understood (Canto *et al*., 1997). Rather, recombinants between the two

types of RNA 3 were selected and initiated the systemic infection. These recombination events occurred in the intergenic region, within the capsid protein subgenomic promoter region (T. Canto and P. Palukaitis, unpublished results).

C. Plant-to-Plant Movement

Cucumoviruses are transmitted through the seed, although to varying extents (Edwardson and Christie, 1991). Seed transmission has been described for all three virus species and occurs in some but not all plant species of their host range. Variation in the efficiency of seed transmission of CMV has been reviewed previously (Palukaitis *et al*., 1992), with efficiencies varying from a fraction of 1% to more than 50%. Numerous papers have been published since that have reported the incidence and prevalence of seed transmission of cucumoviruses; however, few have shed much light on the mechanism of transmission. Virus may be present in the embryo, endosperm, and seminal integuments, as well as in the pollen (Yang *et al*., 1997). In the case of CMV, RNA 1, and presumably the 1a protein, has some role in affecting the incidence of seed transmission (Hampton and Francki, 1992). This field remains insufficiently researched, and no progress has been made in understanding the mechanism of transmission of cucumoviruses via seeds.

The vector transmission of cucumoviruses occurs by aphids in a nonpersistent manner. CMV is transmitted by over 80 species of aphids, with *Myzus persicae* and *Aphis gossypii* being two efficient and important ones. TAV is transmitted by 22 species of aphid and PSV by 5 species (Edwardson and Christie, 1991). The transmission efficiency varies with several factors, particularly the specific combination of virus isolate and aphid species (Chen and Francki, 1990; Gera *et al*., 1979; Simons, 1958) and the accumulation level of virus particles in the source leaf (Banik and Zitter 1990; Escriu *et al*., 2000a; Normand and Pirone, 1968; Pirone and Megahed, 1966). Strains have been described that are transmitted very poorly by one or more species of aphids (Badami, 1958; Gera *et al*., 1979; Mossop and Francki, 1977; Normand and Pirone, 1968). Some of these strains have been used to show that the capsid protein is the only virus determinant of aphid transmissibility and vector specificity (Chen and Francki, 1990; Gera *et al*., 1979). The aphid transmission of poorly transmitted isolates can be complemented from mixed infections with efficiently transmissible ones, which may also result in the appearance of reassortant genomes (Perry and Francki, 1992).

Some progress has been made in characterizing some of the parameters involved in the aphid transmission of CMV. The interpretation of data has been helped considerably by the elucidation of the three-dimensional structure of CMV (Smith *et al*., 2000). Amino acid sequences in the capsid protein have been identified that influence the efficiency of aphid transmission. A detailed analysis of the determinants of aphid transmission has been made by the analysis of chimeric and mutant strains of CMV derived from efficiently and poorly transmitted isolates. Amino acids at positions 129 and 162 of the capsid protein determined transmissibility by *A. gossypii*, whereas transmissibility by *M. persicae* was determined by these amino acids as well as those at positions 25, 168, and 214 (Perry *et al*., 1994, 1998). Amino acid sequences (191–197) in the β strand H-I loop on the surface of CMV virions (Smith *et al*., 2000) were shown to be important for efficient aphid transmission, although not greatly affecting either the infectivity or the assembly of CMV (Liu *et al*., 2002). The efficiency of transmission was also affected by amino acid changes at additional positions, more so for transmission by *M. persicae* than for transmission by *A. gossypii* (Perry *et al*., 1994, 1998). The amino acid positions that determined transmission either were exposed on the outer surface of the capsid (e.g., 129) or lay in the inner face (e.g., 162) (Smith *et al*., 2000). Hence, their effect could be either by direct interaction with the aphid mouth parts or by affecting particle stability.

Interestingly, the transmissible phenotype of CMV appears to be very stable: seven field isolates of CMV retained the ability to be transmitted by *M. persicae* and *A. gossypii* after 24 mechanical passages, and only one of them lost the ability to be transmitted by *M. persicae*, but not by *A. gossypii*, after further passage (Ng and Perry, 1999). This is in sharp contrast with reports of loss of transmissibility upon the passage of potyviruses and other nonpersistently transmitted viruses that rely on a helper factor for vector transmission (reviewed by Pirone and Blanc, 1996). The loss of transmissibility of CMV could be selected against because it might result in a less stable particle, as shown for mutants at position 162 (Ng *et al*., 2000), or affect other aspects of the virus life cycle. For example, the proline at position 129, which is conserved in all transmissible isolates, is necessary for efficient cell-to-cell movement in certain host plants (Wong *et al*., 1999).

In addition to factors associated with the aphid and the virus, the transmission of cucumoviruses may also be determined by factors associated with the host plant, as shown by the resistance to transmission of CMV in melon plants (*Cucumis melo*) containing the *Vat* gene. This gene confers resistance to neither the aphids nor the virus, but

specifically to virus transmission (Lecoq *et al*., 1979). Aphids can acquire CMV from mechanically inoculated plants containing the *Vat* gene, but cannot transmit it to these plants. *Vat* resistance is aphid specific, as it inhibits transmission by *A. gossypii* but not by *M. persicae*. The probing and feeding behavior of these two aphid species in melon plants containing the *Vat* gene is different, but the observed difference explains only in part the aphid specificity of the *Vat* gene (Chen *et al*., 1997), showing the complexities of the process of CMV transmission by its aphid vectors.

VI. Resistance and Host Responses

A. Host-Derived Resistance

Resistance to infection by CMV and PSV has been described for a number of plants species (reviewed by Edwardson and Christie, 1991; Fraser, 1986, 1990). In many cases, the resistance was multigenic, and recessive, but usually was not characterized further (e.g., Caranta *et al*., 2002; Chaim *et al*., 2001; Grube *et al*., 2000). In other cases, the phenotype was better characterized, but virtually nothing is known about the genetics of resistance (Celebi *et al*., 1998; Dufour *et al*., 1989; Gal-On *et al*., 1994; Kobori *et al*., 2000; Lakshman and Gonsalves, 1985; Roossinck, 1991; Ryu *et al*., 1998a; Takeshita *et al*., 1998, 2001; Takahashi *et al*., 2000; Thompson and García-Arenal, 1998; Valkonen *et al*., 1995; Wong *et al*., 1999). However, in a few examples, resistance was shown to be due to the effects of a single gene (e.g., Jones and Latham, 1997; Stamova and Chetelat, 2000). In cowpea, a single, dominant gene for resistance to CMV has been designated *Cry* (Nasu *et al*., 1996). Resistance conferred by this gene induced a hypersensitive response, which led to a restriction of virus infection to a small local lesion. Resistance breaking strains of CMV, vis-à-vis this gene, occur naturally, and the elicitor of the hypersensitive response has been mapped to the sequences within the 2a polymerase gene (Karasawa *et al*., 1999; Kim and Palukaitis, 1997). The resistance gene has also been mapped (Chida *et al*., 2000) and may be of the NB-LRR type of resistance gene involved in induction of hypersensitive responses to other pathogens (Dangl and Jones, 2001). In *Arabidopsis thaliana*, two single, independent, recessive, and resistance genes have been described: *cum1-1* and *cum2–1* for resistance to CMV (Yoshii *et al*., 1998a, 1998b). Both genes inhibited Y-CMV accumulation in inoculated and systemically infected leaves, and thus probably

affected cell-to-cell movement and also possibly long-distance movement. The *cum2-1* gene also inhibited movement of the *Tombusvirus Turnip crinkle virus* (Yoshii *et al*., 1998b). Curiously, the *cum2-1* gene did not inhibit the cell-to-cell or long-distance movement of Fny–CMV (S. Naito and J.P. Carr, personal communication). Differences between resistance mediated against CMV strains were also observed in *A. thaliana* ecotype C24, where Y-CMV, but not O-CMV, induced a hypersensitive response that has been mapped to a single, dominant gene designated *RCY1* (Takahashi *et al*., 1994, 2001). The capsid protein of Y–CMV was shown to be the elicitor of the hypersensitive response, which restricted CMV infection to a local lesion (Takahashi *et al*., 2001). The *RCY1* gene has now been isolated and characterized (Takahashi *et al*., 2002). This gene encodes a 104-kDa protein with the characteristic domains of a CC-NB-LRR-type resistance gene product. *RCY1* is allelic to the *A. thaliana* genes *RPP8*, conferring resistance to *Peronospora parasitica* biotype *Emco5*, and *HRT*, conferring resistance to *Turnip crinkle virus*. The resistance conferred by *RCY1* operates via two pathways: one involving SA and ethylene signaling and a second novel signaling mechanism (Takahashi *et al*., 2002). Resistance in cucumber cv. China (Kyoto) was determined by at least two recessive genes (Boulton *et al*., 1985). Resistance to systemic movement in pepper was inherited as a dominant trait and was determined by seven genomic regions, including one major effect and several minor effect QTLs (Caranta *et al*., 2002).

In some other instances, the nature of the resistance has been analyzed in more detail. Plants of *Lycopersicon chmielevskii* and *Lycopersicon hirsutum*, resistant to CMV by mechanical inoculation, were susceptible to graft inoculation (Abad *et al*., 2000). In pepper (*Capsicum annuum*; Dufour *et al*., 1989), Japanese radish (*Raphanus sativus*; Takeshita *et al*., 1998), and bottle gourd (*Lagenaria siceraria*; Takeshita *et al*., 2001), resistance to CMV was shown to be due to a blockage of long-distance movement, whereas in maize (*Zea mays*; Ryu *et al*., 1998a) and pea (Kim and Palukaitis, 1993), resistance was found to be due to a block in cell-to-cell movement. In cucumber cv. China (Kyoto), resistance to CMV was shown to be due to an inhibition of replication (Maule *et al*., 1980), whereas in *Cucumis figarei*, resistance was shown to be due to inhibition of movement into the vascular cells (Kobori *et al*., 2000). Finally, resistance to systemic accumulation of virus has also been observed to occur in susceptible plants as a consequence of plant aging (García-Ruiz and Murphy, 2001). Older plants were also more resistant to infection by CMV with a deletion of the 2b gene (Ji and Ding, 2001).

B. Pathogen-Derived and Transgene-Mediated Resistance

Various viral genes and segments have been expressed in transgenic plants and conferred resistance against CMV. Many examples of capsid protein-mediated resistance have been described, in tobacco, as well as in other crops (Table II). The extent of resistance varied considerably, both with different constructs and in different hosts. In some cases, resistance was not obtained (Table II). In others cases, resistance was specific to strains in the same subgroup from which the donor capsid protein gene originated. Broad resistance to CMV strains in subgroups I and II was achieved in only a few cases. The mechanisms of resistance may have been protein mediated in some cases, but most examples probably were forms of RNA-mediated resistance, even though antisense RNA to the capsid protein gene was not very successful (Cuozzo *et al*., 1988). Sequences derived from the 5′- or 3′-nontranslated regions did not, in general, show resistance to CMV infection, except in one line of one early example (Rezaian *et al*., 1988). This was also probably the first case of an RNA-mediated resistance to a virus.

Resistance to CMV was described in transgenic tobacco plants expressing replicons of CMV RNAs 1 plus 2 (Table II), but not by expression of CMV RNAs 1 or 2 alone (Suzuki *et al*., 1996). The mechanism of resistance appeared to operate more effectively against RNA as an inoculum than against virions (Suzuki *et al*., 1996). In contrast to the aforementioned example, resistance mediated by the expression of intact and defective polymerase genes as well as a full-length RNA 1 has been described (Table II). This difference in detection of resistant transgenic plants may be due to the number of transgenic lines analyzed, as such resistance did not occur with a high frequency (Anderson *et al*., 1992; Gal-On *et al*., 1998; P. Palukaitis *et al*., unpublished data; Wintermantel and Zaitlin, 2000). The mechanisms of replicase-mediated resistance conferred by expression of an intact RNA 1 or a defective RNA 2 have some features in common. Both types of transgenic plants blocked replication of the target RNA (Canto and Palukaitis, 2001; Carr *et al*., 1994) and both blocked long-distance movement through the phloem (Canto and Palukaitis, 1998, 2001; Carr *et al*., 1994; Wintermantel *et al*., 1997). Both resistance mechanisms were also subgroup sequence specific (Canto and Palukaitis, 1998; Hellwald *et al*., 2001; Zaitlin *et al*., 1994). However, the specific target sequences in RNA 2 for breakage of the inhibition against virus replication were different from those specifying an inhibition of virus movement (Hellwald and Palukaitis, 1995).

Satellite RNAs have also been used to demonstrate pathogen-derived resistance (Table II). In these cases the satellite RNA reduced virus accumulation and attenuated virus-induced pathogenicity rather than inhibiting virus infection. A combination of capsid protein- and satellite RNA-mediated resistance proved more effective than either alone (Yie *et al*., 1992). Satellite RNA-mediated resistance also has been demonstrated by prior "vaccination" of plants (Crescenzi *et al*., 1993; Gallitelli *et al*., 1991; Montasser *et al*., 1991; Montasser *et al*., 1998; Sayama *et al*., 1993; Tien *et al*., 1987; Wu *et al*., 1989).

Resistance against CMV infection has also been demonstrated by expressing the transgenically defective 3a movement protein (Zhang *et al*., 1999), although whether this resistance is broad or limited to strains of CMV in the same subgroup as the transgene donor strain remains to be determined. In other instances, using either a transgene expressing an N-terminally mutated 3a protein (R.G. Dietzgen, personal communication) or transgenes expressing any of four dysfunctional movement proteins (F. Cillo and P. Palukaitis, unpublished results), no resistance was observed in transgenic tobacco or *Nicotiana benthamiana*, respectively.

Resistance or partial protection against symptom induction has also been achieved to varying extents by the transgenic expression of ribozymes targeted against individual CMV RNAs or by the use of a combination of satellite RNA plus a ribozyme (Table II). In addition, resistance or protection to CMV has occurred to varying extents by transgenic approaches that were not specific to CMV (Table II). The expression of an antisense gene to tobacco *S*-adenosylhomocysteine hydrolase, to inhibit the methylation of viral 5′ cap structures, showed some effect at inhibiting the systemic infection of a range of viruses (Masuta *et al*., 1995), as did the expression of a gene for the pokeweed (*Phytolacca americana*) antiviral protein, which degrades RNAs (Lodge *et al*., 1993). The interferon-regulated 2-5A system was tested for its ability to inhibit infection by a range of plant virus by expression of genes for human 2′,5′-oligoadenylate synthetase and 2-5A-dependent ribonuclease (RNase L). This system also showed resistance against systemic infection by CMV (Ogawa *et al*., 1996). The effectiveness of these various forms of resistance has not been evaluated extensively.

The question arises as to whether pathogen-derived resistance will be comparable to, inferior to, or superior to conventional resistance. This question has not been addressed yet in substantial detail to make an evaluation. However, in one study, cucurbit species containg either conventional or transgenic resistance to CMV were found to retain

TABLE II
TRANSGENE-MEDIATED RESISTANCE TO CUCUMOVIRUSES

RNA/gene[a]	Donor strain[b]	Host species	Resistance phenotype	Reference[c]
Capsid protein (CP)	D (IA)[d]	Tobacco	NS[e]	1
CP	D (IA)	Tomato	NS	2
CP	C (IA)	Tobacco	NS, delay, less disease	3,4
CP	C (IA)	Cucumber	NS or delay	5
CP	C (IA)	Squash	NS or less disease	6
CP	WL (II)	Tobacco	NS	7
CP	WL (II)	Melon	Delay	8
CP	WL (II)	Tomato	NS	9–11
CP	O (I)	Tobacco	NS	12,13
CP	O (I)	Cucumber	NS or tolerance	14
CP	Y (IA)	Tobacco	NS or delay	15–17
CP	Y (IA)	Melon	NS	18
CP	SB91/366 (I)	Tobacco	NS or delay	19
CP	CP91/367 (I)	Tobacco	NS or delay	19
CP	ZU (I)	Tomato	NS	20
CP	PG (II)	Tomato	NS	2,21
CP	22 (I) + PG (II)	Tomato	NS	2,21
CP	Wem (II)	Tobacco	NS	22
CP	R (II)	Tobacco	Recovery	23
CP	I17F (IA)	Tobacco	No resistance	23
CP	Kor (IA)	Pepper	Delay	24
CP	As (I)	Tobacco	NS or delay	25
SatRNA[f]	I17N (IA)[g]	Tobacco	NS	26,27
SatRNA	1 (IA)	Tobacco	NS	12
SatRNA	T73	Tomato	NS or less disease	28
SatRNA	T73	Tobacco	NS	29
SatRNA	S (II)	Tomato	Tolerance	30,31
SatRNA	I17N (IA)	Petunia	Less disease	32
SatRNA	I17N (IA)	Pepper	Delay, less disease	33
CP + SatRNA	O + 1	Tobacco	NS	12,34
R3 ribozyme[h]		Tobacco	Delay	17

(*continues*)

TABLE II (*continued*)

RNA/gene[a]	Donor strain[b]	Host species	Resistance phenotype	Reference[c]
R1/R2 ribozyme		Tobacco	Delay	35
SatRNA + ribozyme		Tobacco	NS or delay	35
3a[i]		Tobacco	NS	36
2a[j]	Fny (IA)	Tobacco	NS, delay, or no resistance	4, 37–40
2a[i]	Fny (IA)	Tomato	NS or delay	41
1a	Fny (IA)	Tobacco	NS or no resistance	42
R1 + R2[k]	Y (IA)	Tobacco	NS	43
PAP[l]		Tobacco	NS	44
Anti-SAHH[m]		Tobacco	NS or delay	45
2-5A + RNase L[n]		Tobacco	NS	46

[a] Source of the RNA or transgene used for transformation.

[b] Strain of CMV from which the viral-derived transgene originated.

[c] 1, Cuozzo *et al*. (1998); 2, Kaniewski *et al*. (1999); 3, Quemada *et al*. (1991); 4, Singh *et al*. (1998); 5, Gonsalves *et al*. (1992); 6, Tricoli *et al*. (1995); 7, Namba *et al*. (1991); 8, Gonsalves *et al*. (1994); 9, Xue *et al*. (1994); 10, Provvidenti and Gonsalves (1995); 11, Fuchs *et al*. (1996); 12, Yie *et al*. (1992); 13, Nakajima *et al*. (1993); 14, Nishibayashi *et al*. (1996); 15, Okuno *et al*. (1993b); 16, Okuno *et al*. (1993a); 17, Nakamura *et al*. (1995); 18, Yoshioka *et al*. (1993); 19, Rizos *et al*. (1996); 20, Gielen *et al*. (1996); 21, Murphy *et al*. (1998); 22, Hackland *et al*. (2000); 23, Jacquemond *et al*. (2001); 24, Shin *et al*. (2002); 25, Ryu *et al*. (1998b); 26, Harrison *et al*. (1987); 27, Jacquemond *et al*. (1988); 28, Saito *et al*. (1992); 29, Masuta *et al*. (1994); 30, McGarvey *et al*. (1994); 31, Stommel *et al*. (1998); 32, Kim *et al*. (1995); 33, Kim *et al*. (1997); 34, Yie *et al*. (1995); 35, Kwon *et al*. (1997); 36, Zhang *et al*. (1999); 37, Anderson *et al*. (1992); 38, Zaitlin *et al*. (1994); 39, Hellwald and Glenewinkel (1999); 40, Wintermantel and Zaitlin (2000); 41, Gal-On *et al*. (1998); 42, Canto and Palukaitis (1998); 43, Suzuki *et al*. (1996); 44, Lodge *et al*. (1993); 45, Masuta *et al*. (1995); 46, Ogawa *et al*. (1996).

[d] Subgroup of the CMV donor strain.

[e] No symptoms (in some cases, but not always, associated with reduced virus titers).

[f] Satellite RNA.

[g] Strain and subgroup origin of CMV satellite RNA.

[h] Ribozyme targeted against the RNA specified.

[i] Defective gene.

[j] Defective and nondefective gene.

[k] RNA 1 and RNA 2 replicon.

[l] Gene for pokeweed antiviral protein from *Phytolacca americana*.

[m] Antisense gene to tobacco *S*-adenosylhomocysteine hydrolase.

[n] Genes for human 2′,5′-oligoadenylate synthetase and 2-5A-dependent ribonuclease.

TABLE III
GENETIC MAPPING OF TRAITS

RNA	Gene	Position	Virus	Phenotype	Reference[b]
1	1a?	NE[a]	CMV	Hypersensitive response in tobacco	1
1	1a?	NE	CMV	Rapid local and systemic movement in squash	2
1	1a?	NE	CMV	Seed transmission in legumes	3
1	1a	173	CMV	Replication (ts)[c] in melon	4
1	1a	978	CMV	Replication of satellite in squash	5
1	1a?	NE	CMV	Replication of satellite	6
1	1a?	NE	PSV	Replication of satellite	7
2	2a	631/641	CMV	Hypersensitive response in cowpea	8,9
2	2b	NE	CMV	Hypervirulence; systemic movement	10–13
2	2b	NE	TAV	Hypervirulence	14,15
2	2b	NE	CMV	Suppression of gene silencing	11,15
2	NE	NE	CMV	Host range and/or pathology responses	1,16,17
3	3a	20/21	CMV	Limited movement between epidermal cells	18,19
3	3a	20/21	CMV	Tubule formation from protoplasts	19
3	3a	51	CMV	Systemic movement in bottle gourd	20
3	3a	51+240	CMV	Systemic movement in cucurbits	21
3	3a	60	CMV	Systemic movement (ts) in tobacco	22
3	3a	97/98; 144/145; 156/158; 191/192[d]	CMV	Cell-to-cell movement	22
3	3a	174–233	CMV	RNA-binding domain	23
3	3a	247–279	CMV	Capsid protein-dependent movement[e]	24
3	3a	nt 100[f]	TAV	RNA accumulation	25
3	CP	14–20	CMV	Virion assembly	26

(*continues*)

TABLE III (*continued*)

RNA	Gene	Position	Virus	Phenotype	Reference[b]
3	CP	26–40	CMV	Virion assembly	27
3	CP	36	CMV	Hypersensitive response on *Nicotiana* sp.	28
3	CP	81/166/173[g]	CMV	Virion assembly	27
3	CP	111/124	CMV	Green mosaic on tobacco	29
3	CP	129	CMV	Chlorosis or veinal necrosis on tobacco	30,31
3	CP	129	CMV	Virion assembly	31
3	CP	129	CMV	Systemic movement in bottle gourd	20
3	CP	129+162	CMV	Local and systemic movement in maize	32
3	CP	129+214	CMV	Local and systemic movement in squash	33
3	CP	129+162+168	CMV	Aphid transmission by *Aphis gossypii*	34
3	CP	25+129+162+ 168+214	CMV	Aphid transmission by *Myzus persicae*	34,35
3	CP	138	CMV	Virion assembly	31
3	CP	144	CMV	Virion assembly	31
3	CP	147	CMV	Virion assembly	31
3	CP	162	CMV	Virion stability	36
3	CP	193	CMV	Stunting in *Nicotiana glutinosa*	37
3	CP	NE	CMV	Systemic infection in *Cucumis figarei*	38
3	CP	NE	TAV	Systemic infection in cucumber	39,40
3	RNA	5′ 618 nt	CMV	Necrotic etched ring in inoculated leaf	41
3	NE	NE	CMV	Host range and/or pathology responses	16,17

[a] Not established.

[b] 1, Lakshman and Gonsalves (1985); 2, Gal-On *et al*. (1994); 3, Hampton and Francki (1992); 4, Bao *et al*. (1999); 5, Roossinck *et al*. (1997); 6, McGarvey *et al*. (1995); 7, Hu *et al*. (1998); 8, Kim and Palukaitis (1997); 9, Karasawa *et al*. (1999); 10, Ding *et al*. (1995b); 11, Brigneti *et al*. (1998); 12, Shi *et al*. (2002); 13, Soards *et al*. (2002); 14, Ding *et al*. (1996); 15, Li *et al*. (1999); 16, Palukaitis *et al*. (1992); 17, Takeshita *et al*. (1998); 18, Canto and Palukaitis (1999a); 19, Canto and Palukaitis (1999b); 20, Takeshita *et al*. (2001); 21, Kaplan *et al*. (1997); 22, Li *et al*. (2001); 23, Vaquero *et al*. (1997); 24, Nagano *et al*. (2001); 25, Moreno *et al*. (1997b); 26, Schmitz and Rao (1998); 27, Kaplan *et al*.

(*continues*)

their resistance in the field in California, even though preexisting resistance breaking isolates were also present in neighboring fields (Lin *et al*., 2003).

C. Host Range and Pathogenicity Determinants

Sequences involved in host range or pathogenicity have been mapped to each of the five known genes of CMV, as well as to the RNA sequence. Considerable amounts of data have been assimilated in this area in the last decade. In many cases, specific amino acids have been identified that influence these phenotypes (Table III). The mapping of such sequences has allowed some dissection of the roles of particular domains of the five cucumoviral-encoded proteins in the processes of replication, movement, and encapsidation. Moreover, sequences pleotropic for various interactions have also been identified. This is particularly true of sequences in the capsid protein (Table III). For example, amino acid 129 of the capsid protein has been identified as being involved in affecting no less than six interactions (Table III). For the various interactions between viral-encoded gene products and host factors affected by the mutations listed in Table III, neither the cellular specific sites of interaction nor the specific host factors involved in the interactions have been determined, although sites of localization of the various CMV-encoded proteins have been delineated (see Sections III and IV,A), as have various barriers to infection (see Section V,A). The former also applies to capsid protein sequences and the nature of their interaction with aphid stylet components.

D. Host Responses

Analysis of the host responses to infection by cucumoviruses can be considered in several specific classes: resistance responses, general pathological responses, and specific responses to the presence or action

(1998); 28, Takahashi *et al*. (2000); 29, Sugiyama *et al*. (2000); 30, Shintaku *et al*. (1992); 31, Suzuki *et al*. (1995); 32, Ryu *et al*. (1998a); 33, Wong *et al*. (1999); 34, Perry *et al*. (1994); 35, Perry *et al*. (1998); 36, Ng *et al*. (2000); 37, Szilassy *et al*. (1999); 38, Saitoh *et al*. (1999); 39, Taliansky and García-Arenal (1995); 40, Thompson and García-Arenal (1998); 41, Zhang *et al*. (1994a).

[c] Temperature sensitive.

[d] All four pairs of mutants individually affected virus movement.

[e] Deletion rendered cell-to-cell movement capsid-protein independent.

[f] Nucleotide sequence alteration affecting RNA accumulation.

[g] Not delimited further.

of particular viral gene products. The last can be with respect to either resistance/susceptibility interactions or pathology.

Infection of tobacco by CMV usually produces a mosaic in which the light green (or yellow) areas contain virus, and the dark green "islands" contain very little virus (Loebenstein *et al*., 1977). The green island tissue in tobacco cv. Xanthi nc contains an inhibitor of CMV replication, which was of similar size and was related serologically to the inhibitor of virus replication obtained from tobacco cv. Samsun NN infected with TMV (Gera and Loebenstein, 1988). The gene encoding this inhibitor of TMV replication has been cloned, and the gene was expressed only after TMV infection in tobacco cv. Samsun NN plants, but not in tobacco cv. Samsun nn plants (Akad *et al*., 1999). The 21.6-kDa protein expressed from this gene inhibited TMV replication in leaf disk assays (Akad *et al*., 1999), but has not been evaluated further for inhibition of CMV replication. It is unlikely that this inhibitor is the same one described as a "symptom-delaying component" (Ohki *et al*., 1991) since the latter is sensitive to ribonuclease A and may be related to one of the CMV RNA species.

Resistance to virus infection manifested by a hypersensitive response in plants also induces a general systemic acquired resistance against a broad spectrum of pathogens (reviewed by Metraux *et al*., 2002). Salicylic acid (SA) has been shown to be a signaling molecule for the induction of defense reactions at the site of infection as well as for systemic-acquired resistance (reviewed by Dempsey *et al*., 1999; Murphy *et al*., 1999). Pretreatment of tobacco plants with SA did not inhibit either CMV replication or cell-to-cell movement, but rather delayed the long-distance movement of CMV (Murphy and Carr, 2002; Naylor *et al*., 1998). The delay in long-distance movement could be prevented by application of salicylhydroxamic acid, an inhibitor of a novel defense signal transduction pathway (Naylor *et al*., 1998). The CMV 2b protein has been shown to interfere with the SA-mediated defense response (Ji and Ding, 2001).

Cytopathological responses to infection by cucumoviruses in susceptible and resistant plants have been reviewed previously (Francki *et al*., 1985; Martelli and Russo, 1985; Palukaitis *et al*., 1992). A more detailed analysis of changes in physiology and enzyme levels in the susceptible interaction of CMV in marrow (also known as squash; *C. pepo*) has been done (Técsi *et al*., 1994a, 1994b, 1996). These studies established that while metabolic alterations occurred or did not for various enzymes involved in photosynthesis, respiration, or biosynthesis

within the infected lesion, overall changes in enzyme levels were misleading, as different changes occurred in different zones of the expanding chlorotic lesion. Several defined zones appeared in the chlorotic lesion between 4 and 6 dpi: (i) an outer zone that had elevated levels of virus replication, photosynthesis, and protein synthesis, but low levels of starch; (ii) an adjacent zone with high biosynthetic activity throughout, as well as high photosynthetic activity, low levels of starch, and high protein synthesis activity at the outer edge, but with low photosynthetic activity, high starch accumulation, and low protein synthesis activity at the inner edge; (iii) an inner zone of lower starch content with high biosynthetic activity and respiration, but very low photosynthetic activity, and showing chlorosis by 4 dpi; and (iv) a small group of infected cells that first showed starch accumulation at 2 dpi, but showed a decrease in starch accumulation by 6 dpi. Virus accumulated in all zones, but replication was active only in the outmost zone (i) by 6 dpi (Tésci *et al*., 1996). CMV infection in the inoculated leaves of tobacco also appeared to follow a pattern of temporally and spatially dependent host responses, as was evident by the formation of necrotic-etched rings (Zhang *et al*., 1994a).

Further analysis of mRNA levels for genes encoding various enzyme activities altered during lesion formation in squash showed that gene expression in the lesion was downregulated for several genes (Havelda and Maule, 2000). These genes encoded either various enzymes analyzed previously (Tésci *et al*., 1996) or host response proteins (i.e., heat shock protein 70 and catalase). Interestingly, expression from these last two genes, as well as the gene encoding the $NADP^+$-dependent malic enzyme, also showed upregulation outside the lesion, although for different distances (numbers of cells) from the lesion (Havelda and Maule, 2000). Thus, CMV infection can alter enzyme activities by regulation of the levels of gene expression. Moreover, there is a signal that extends beyond the site of virus replication that regulates the expression of some genes.

Coordinated changes in gene expression in *A. thaliana* plants infected by CMV and four other viruses were analyzed by microarray hybridization (Whitham *et al*., 2003). In all, 114 unique genes were induced in response to infection by all five viruses. Most of the genes induced were involved in one of the following processes: metabolism; signal transduction; protein destination; transcription; cellular organization; or defense, death, and aging. About 20% of the genes induced could not be classified. In general, infection by CMV showed a

transient pattern of induction of a number of defense-related response genes. Most of these genes induced at 2 days after infection were not induced at 3–5 dpi, whereas a new set of genes induced at 4 dpi were mostly downregulated again by 5 dpi (Whitham *et al*., 2003). It is not clear to what extent these changes in expression profiles relate to the spatial distribution of the virus with time in the infected tissues.

Infection of tobacco by Y–CMV induced local chlorotic spots on the inoculated leaves, as well as systemic chlorosis. The chlorotic spots were produced on the inoculated leaves by 5 dpi (Takahashi *et al*., 1991). Analysis of proteins present in the chlorotic spots showed that six proteins decreased and three increased in level. Two proteins that decreased were isoforms (22 and 23 kDa) and were related to a 23-kDa oxygen-evolving complex protein in photosystem II from spinach (*Spinacia oleracea*; Takahashi *et al*., 1991). The 22-kDa isoform originated from the tobacco parent *Nicotiana sylvestris*, and the 23-kDa isoform originated from the other tobacco parent, *Nicotiana tomentosiformis* (Takahashi *et al*., 1991). The level of the 33-kDa protein of the oxygen-evolving complex was not affected during the first 5 dpi, although the oxygen-evolving activity did show some decrease (Takahashi and Ehara, 1992). The genetic analysis of chlorotic spot development in this tobacco cultivar (Ky57) showed that two recessive genes were required for interaction with the capsid protein of Y–CMV, which led to chlorosis in the inoculated leaves. These genes were closely linked or identical to the recessive *yb* genes (*yb1* and *yb2*), associated with reduced chlorophyll presence in the stem (Takahashi and Ehara, 1993). The nature of the interaction(s) of the capsid protein of either Y–CMV or any of several other strains of CMV inducing chlorosis on tobacco, with tobacco genes or proteins that leads to reductions in the levels of the 22-kDa and 23-kDa proteins, remains unknown.

Infection of melon by CMV resulted in a sharp increase in sucrose accumulation in sap in the infected leaves (Shalitin and Wolf, 2000). A similar, although less pronounced, effect was observed in melon expressing the CMV 3a movement protein from a virus vector (Shalitin *et al*., 2002). This is believed to be due to an alteration in the mode of phloem loading in such plants. In tobacco, transgenic expression of the CMV 3a movement protein also caused a reduction in the levels of soluble sugars and starch and a reduced root-to-shoot mass ratio. However, this response was cultivar specific and perhaps related to the level of expression of the 3a movement protein (Shalitin *et al*., 2002).

VII. Variability and Evolution

A. Genetic Diversity and Taxonomy

Data on host range, symptomatology, immunology, and molecular analysis of the genomic RNAs demonstrate that isolates of CMV are extremely heterogeneous. This led to the early classification of CMV strains into two subgroups based on different criteria (reviewed in Palukaitis *et al*., 1992), now named subgroup I and subgroup II according to molecular analyses of the genomic RNAs (Owen and Palukaitis, 1988). The percentage identity in the nucleotide sequence between pairs of isolates belonging to each of these subgroups ranges from 69 to 77%, depending on the RNA species and the pair of isolates being compared. The dissimilarity in sequence is highest for RNA 2 (Table IV). The percentage identity among isolates of subgroup II is higher than 96%, and among isolates in subgroup I the percentage identity is higher than 88%. This shows that subgroup I is more heterogeneous than subgroup II. [It should be pointed out that the complete nucleotide sequence is available for nine isolates of subgroup I vs four of subgroup II (Table IV).] Within subgroup I, analysis of the RNA 3 open reading frames shows that a group of closely related isolates forms a monophyletic cluster, named subgroup IA (isolates Mf, Leg, Fny, and Y in Fig. 2). The rest of the subgroup I isolates are included in nonmonophyletic subgroup IB. Rearrangements, deletions, and insertions in the 5′-noncoding region of RNA 3 correlate with this division of subgroup I and might be the basis of the origin of the subdivision (Roossinck *et al*., 1999). Interestingly, analysis of RNA 2 open reading frames shows that strains in both IA and IB constitute monophyletic groups, whereas analysis of RNA 1 open reading frames shows no clear division of subgroup I in IA and IB. This indicates that each of the three genomic RNAs of CMV has a different evolutionary history (Roossinck, 2002). Isolates from subgroups IA and II have been found all over the world, but with two exceptions, isolates of subgroup IB are predominantly from east Asia. The exceptions are isolate Tfn from Italy, which most probably is a recent introduction from Asia (Gallitelli, 2000), and two field isolates from California (Lin *et al*., 2003).

The number of isolates characterized biologically, serologically, and molecularly is much smaller for TAV and PSV than for CMV. Genetic heterogeneity of TAV isolates appears to be small, regardless of host origin and symptomatology. The percentage nucleotide sequence identity is above 99% for RNAs 1 and 2 of TAV, and may drop to 91%

TABLE IV
PERCENTAGE NUCLEOTIDE SEQUENCE IDENTITY FOR THE FIVE OPEN READING FRAMES (ORF) OF CUCUMOVIRUSES[a]

ORF		CMV -I	CMV-II	TAV	PSV
1a	CMV-I	88.4–99.7			
	CMV-II	76.7–78.2	98.6–99.1		
	TAV	67.7–68.7	68.0–68.1	99.6	
	PSV	66.1–67.3	66.8–67.4	64.4–65.1	79.3–89.3
2a	CMV-I	89.3–99.7			
	CMV-II	69.4–71.3	96.1–99.3		
	TAV	55.7–56.5	57.7–58.9	99.7	
	PSV	54.7–56.6	56.3–57.8	58.0–58.6	74.9–93.4
2b	CMV-I	68.3–98.5			
	CMV-II	46.2–61.4	95.6–99.0		
	TAV	29.1–38.5	34.2–36.3	100.0	
	PSV	32.9–38.5	31.3–38.8	27.1–49.2	47.4–92.8
3a	CMV-I	92.6–98.9			
	CMV-II	77.0–79.4	98.5–99.5		
	TAV	63.6–65.3	63.6–64.2	99.3	
	PSV	60.8–64.9	63.1–66.4	69.9–71.7	80.7–92.1
CP	CMV-I	91.6–98.5			
	CMV-II	74.9–77.8	98.0–99.5		
	TAV	51.6–52.8	51.7–52.3	99.4	
	PSV	49.6–54.0	50.6–54.4	63.1–67.5	71.1–82.6

[a] Data given are ranges of percentage identity in each two by two comparisons among cucumoviral isolates. In addition to the 18 isolates listed in Table I, isolate S–CMV in subgroup II was included because the nucleotide sequences for all the ORFs are in the database.

for RNA 3 due to tandem duplications in the 3′-noncoding region in some isolates. For PSV, isolates from North America were grouped into two serogroups, named eastern (E) and western (W) (Mink *et al*., 1969). This was supported by competition hybridization analyses (Diaz-Ruiz and Kaper, 1983). Later, sequence analyses supported the division of PSV isolates into two subgroups, named I (E) and II (W) (Hu *et al*., 1997). Characterization of new isolates showed that these subgroups were not limited to North America (Bananej *et al*., 1998; Karasawa *et al*., 1992; Militao *et al*., 1998). PSV isolates from Europe seemed to be more heterogeneous and up to six serogroups were proposed (Richter *et al*., 1987). Molecular hybridization analyses showed that the Robinia strain, previously named *Robinia mosaic virus*

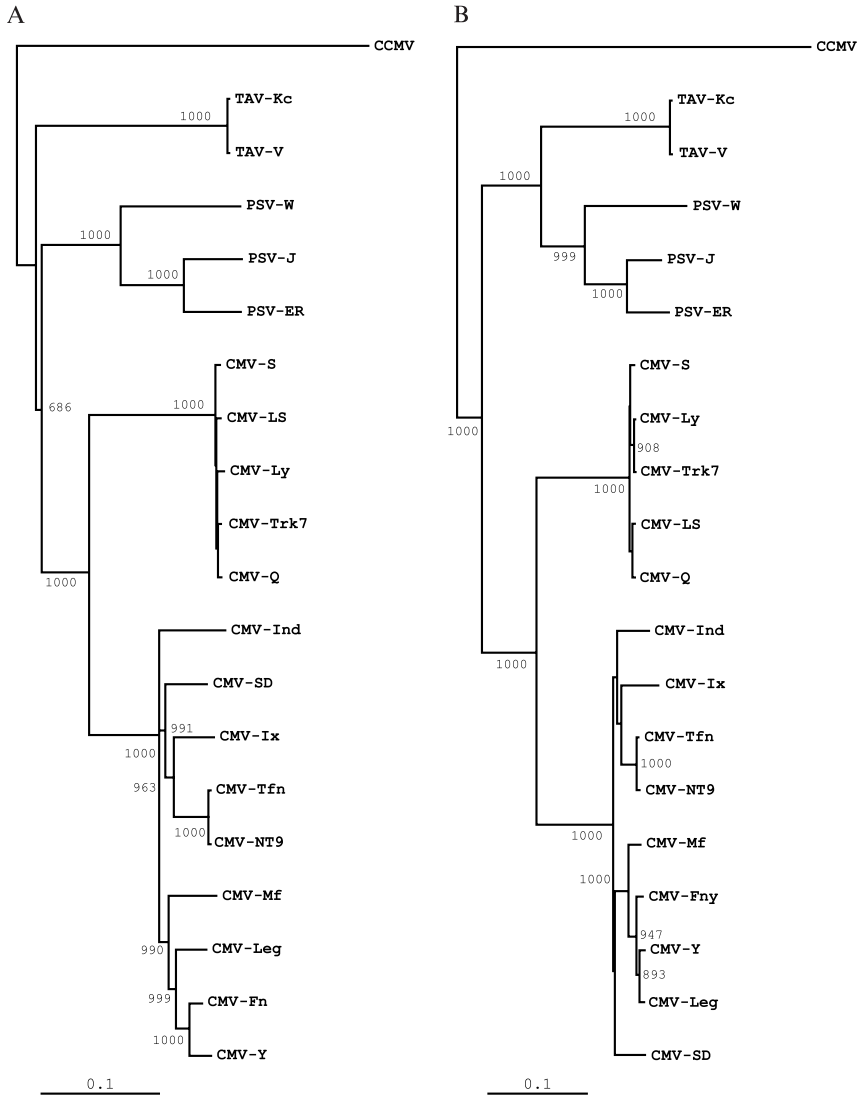

FIG 2. Neighbor-joining phylogenetic trees based on Jukes–Cantor genetic distances derived from the nucleotide sequences of the open reading frames for proteins 1a (A) and 3a (B) of 19 cucumoviral isolates. Bootstrap values after 100 replicates are indicated in the nodes; branch length is proportional to genetic distance. *Cowpea chlorotic mottle virus* (genus *Bromovirus*) was used as an outgroup.

(Schmelzer, 1971), belonged to a different subgroup of PSV (Militao *et al*., 1998). Also, a new subgroup of PSV isolates has been described from China (Zeyong *et al*., 1998). The percentage identity between the sequenced PSV isolate of subgroup II (W isolate) and those in subgroup I was about 79% for RNAs 1 and 3 and about 75% for RNA 2. For the capsid protein gene, the sequence identity among isolates in subgroups I, II, and those from China is about 75% (Zeyong *et al*., 1998). As new isolates are characterized, it seems that the heterogeneity of PSV and the complexity of its taxonomy may be no less than for CMV.

The percentage sequence identity among isolates of the three cucumovirus species varies between 51 and 67%, depending on the RNAs and isolates being compared. The similarity in sequence is highest for RNA 1 and lowest for RNA 3. The open reading frame for the 1a protein is the most conserved between species (~64–69% identity), and the 2b open reading frame is the least conserved (~27–49% identity) (Table IV). Phylogenetic relationships among 19 cucumoviruses are shown in Fig. 2. For all the open reading frames, isolates of each species are in different monophyletic groups. For the open reading frames of RNAs 2 and 3, TAV and PSV isolates form a monophyletic group that is the sister group of CMV, as shown for the open reading frame of the 3a gene in Fig. 2A. This is not the case for the open reading frames of RNA 1, for which the three cucumovirus species are sister to each other, but a closer proximity of CMV–PSV is apparent (Fig. 2B). This shows that the evolutionary history of the three genomic RNAs is different, as noted originally by White *et al*. (1995a). Figure 2 also shows the relationships between the CMV strains discussed earlier.

B. Recombination, Reassortment, and Evolution

Mutation is a primary source of genetic variation in organisms, particularly in RNA viruses (García-Arenal *et al*., 2001). RdRp are error prone, and a consequence is that the replication of a template results in a swarm of sequence variants around a consensus sequence (Domingo and Holland, 1997). The diversity of a CMV population derived from a biologically active cDNA clone was estimated after passage in *N. benthamiana*. The frequency of mutations in the passaged CMV population was higher than for parallel populations of TMV or *Cowpea chlorotic mottle virus* (Schneider and Roossinck, 2000). When the passage experiments were repeated in a range of different host plants, genetic diversity was found not to be a property of the virus,

but changed for each virus-host interaction (Schneider and Roossinck, 2001).

A second source of genetic variation is genetic exchange by recombination or by reassortment of genomic segments. Recombination in viral RNA is considered to be due to a copy choice mechanism. It has been shown that CMV replicase can switch templates *in vitro*, generating recombinant RNAs, albeit with lower frequency than via replicases of two bromoviruses (Kim and Kao, 2001). Recombination has been studied extensively in the bromoviruses, particularly in the 3′-noncoding region (Bruyere *et al*., 2000). The 3′-noncoding region can also be a hot spot for recombination in cucumoviruses. It has been shown that exchange of the 3′-noncoding region between RNA 1 and RNAs 2 and 3 occurs in CMV passaged in tobacco plants transgenic for the RNA 1, as well as in nontransgenic plants, with recombinants being 5–11% of the RNA 1 population (Canto *et al*., 2001). Data indicate that recombinant RNAs 1 were replicated efficiently. Recombinants in the 3′-noncoding region among the three genomic RNAs have also been reported in four CMV isolates infecting alstroemeria. The recombinant RNAs had an increased fitness in this host, but not in tobacco (Chen *et al*., 2002). Recombination at the 3′ end has been also shown in artificially generated and natural reassortants between different cucumoviruses. This recombination resulted in the 3′ end of RNA 3 being homologous to that of RNAs 1 and/or 2 (Fernández-Cuartero *et al*., 1994, Roossinck, 2002; Suzuki *et al*., 2003) and might be favored by secondary structure elements (Suzuki *et al*., 2003). Recombination may lead to an increased fitness of the recombinant RNA (Fernández-Cuartero *et al*., 1994). In experimental mixed infections under conditions that should not select for recombinants, recombinants formed between markers positioned in the two open reading frames of RNA 3 of BMV showed that the frequency could be as high as 0.2 [calculated in García-Arenal *et al*. (2001), from data in Bruyere *et al*., (2000)]. Recombinants in the intergenic region of RNA 3 between CMV isolates belonging to subgroups IA and IB occurred in experimental mixed infections at frequencies below 0.0007 (F. Escriu, A. Fraile, and F. García-Arenal, unpublished data). In natural populations in Spain, these recombinants were found at frequencies of about 0.06, but data indicate they were selected against (Fraile *et al*., 1997). Recombinants between RNA 3 of CMV and TAV have also been detected in mixed infections in tobacco and in conditions of minimal selection pressure for the recombinants. A hot spot for recombination was found in a region of high sequence homology in the 3a gene (Aaziz and Tepfer, 1999). Hence, recombination may be a frequent event between isolates

and species of cucumoviruses in mixed infections and may have an important role in their evolution, depending on its effect on virus fitness. Recombination events in the 5′-noncoding region of CMV RNA 3 may have played a role in the generation of the subgroups of this virus (Roossinck *et al*., 1999).

Reassortment of genomic segments, also called pseudorecombination, is a possible mechanism of evolution of viruses with multipartite genomes. Indeed, it has been proposed that the possibility of reassortment should counter the disadvantages of a divided genome (Chao, 1988). Reassortants that exchange the three genomic RNAs are obtained easily between isolates of a species and accumulate efficiently in experimental conditions. This is also the case for reassortants that exchange RNA 3 between different species (Palukaitis *et al*., 1992). However, in natural CMV populations in Spain, reassortants between isolates of subgroups IA and IB were rare and appeared to be selected against (Fraile *et al*., 1997). This does not mean that reassortment is not important in the evolution of cucumoviruses; as is the case for recombinants, the fate of a reassortant depends on its fitness. Cucumovirus isolates that are natural reassortants between isolates and species have been described (Hu and Ghabrial, 1998; White *et al*., 1995a). Also, phylogenetic analyses prove that reassortment has played an important role in the origin of CMV strains (Roossinck, 2002) and of the cucumovirus species (White *et al*., 1995a).

Selection of a virus variant with diminished relative fitness may be countered by complementation of the defective function by the wild-type virus in mixed-infected cells. Hence, it was shown that a mutant RNA 3 of TAV defective for cell-to-cell movement was maintained to high levels in the virus population by complementation (Moreno *et al*., 1997b).

Analysis of the genetic structure of virus populations may elucidate factors that determine virus evolution. For cucumoviruses, this analysis has been reported only for CMV. Data from different regions of the world show that CMV populations may be highly diverse (e.g., Gallitelli, 2000; García-Arenal *et al*., 2000; Lin *et al*., 2003; Rodríguez-Alvarado *et al*., 1995). A detailed analysis of the population structure of CMV in Spain showed that the genetic composition of the populations varied significantly with the year of isolation and location (Fraile *et al*., 1997). Similarly, a survey of CMV populations in California showed no correlation of the CMV population structure with geographical region, collection date, or plant species (Lin *et al*., 2003). Thus, the "viral populations showed a random fluctuation in composition," as a metapopulation with local extinction and recolonization

(Fraile *et al*., 1997). Interestingly, the population structure of CMV in Spain correlated with that of its main vectors, *Aphis gossypii* and *Myzus persicae* (Martínez-Torres *et al*., 1998), and could be conditioned by the latter. This population structure differs from that of CMV satellite RNA, as reported both in Italy and in Spain (Alonso-Prados *et al*., 1998; Grieco *et al*., 1997), showing different evolutionary dynamics for the helper virus and the satellite RNA.

VIII. Satellite RNA

A. Support Specificity, Sequences, and Structure

Cucumoviruses are helper viruses of small, linear, single-stranded satellite RNAs that depend on the helper viruses for their replication, encapsidation, and transmission. Satellite RNAs have been found in association with isolates of CMV and PSV. Neither CMV–satellite RNAs nor PSV–satellite RNAs can be maintained by the heterologous helper viruses. No satellite RNAs have been found in association with TAV isolates; however, TAV can be a helper virus for CMV–satellite RNAs, but not PSV–satellite RNA (reviewed by Roossinck *et al*., 1992). Satellite RNAs of cucumoviruses have received much attention as model systems for the study of replication, pathogenesis, structure–function relationships, and genetic variation and evolution of plant pathogenic RNAs. Most of the work reported in these areas has been done with CMV–satellite RNA (reviewed in García-Arenal and Palukaitis, 1999). In addition, CMV–satellite RNA has been used as a biocontrol agent for CMV (reviewed by Tousignant and Kaper, 1998; García-Arenal and Palukaitis, 1999).

Satellite RNAs were first described associated with CMV by J. M. Kaper and colleagues in 1977, following work on the etiology of an epidemic of lethal necrosis of tomato in Alsace, France (Marrou *et al*., 1973). It was shown that CMV containing a satellite RNA induced systemic necrosis in tomato (Kaper and Waterworth, 1977). Since then, many variants of CMV–satellite RNA have been characterized (see García-Arenal and Palukaitis, 1999). PSV-associated satellite RNA was described shortly afterward by the Kaper laboratory and was found associated with a PSV isolated from Virginia in 1976 (Kaper *et al*., 1978).

The sequences of more than 100 CMV–satellite RNA variants, found associated with over 65 CMV isolates in both subgroups I and II of CMV, have been reported. Most variants found all over the world

contain 332–342 nucleotides. Some are considerably larger, with 386–405 nt, because of insertions of sequences relative to the satellite RNAs in the smaller class. These larger satellite RNAs have been found in east Asia and Italy, always in association with Asian CMV strains (García-Arenal and Palukaitis, 1999). All analyzed CMV–satellite RNA have a 5′-terminal cap and a 3′-terminal-CCC, the latter of which cannot be aminoacylated (Roossinck *et al*., 1992). Analysis of CMV–satellite RNA variants showed that sequence identity, excluding insertions in the larger satellite RNAs, ranged between 73 and 99% (Fraile and García-Arenal, 1991). Different open reading frames are found in the sequences of CMV–satellite RNAs, although none of them are conserved universally. Translation products have never been found *in vivo*, and mutational analyses showed that these open reading frames probably are not functional. Currently, from these data, it is accepted generally that CMV–satellite RNAs are noncoding RNAs. Therefore, their biological properties must depend on the direct interaction between satellite RNAs and components of their helper viruses and/or host plants. Hence, the secondary and higher-order structure must be important for determining their biological properties. Different secondary structure models have been proposed for CMV–satellite RNAs, based on the accessibility of bases or phosphodiester bonds to different probes. Such analyses were done mostly in solution (Bernal and García-Arenal, 1997; García-Arenal *et al*., 1987; Gordon and Symons, 1983; Hidaka *et al*., 1988; Rodríguez-Alvarado and Roossinck, 1997), but have been done also *in planta* or in virions (Rodríguez-Alvarado and Roossinck, 1997). All these data show that CMV–satellite RNAs are highly structured molecules, with a high percentage of bases (about 50%) involved in base pairing. This could explain the stability and infectivity of these molecules (García-Arenal and Palukaitis, 1999). Data also all support the presence of highly structured regions in the 5′ and 3′ thirds of the molecule, with little structure in the central region. Interestingly, the accessibility of adenines and cytosines to dimethyl sulfate in the central region was different when the reaction was done *in vivo* vs *in vitro* vs in virions (Rodríguez-Alvarado and Roossinck, 1997). Sequence comparisons showed that maintenance of the structural elements is a constraint for CMV–satellite RNA evolution (Aranda *et al*., 1997; Fraile and García-Arenal, 1991). Nevertheless, an unequivocal association of a secondary structure feature with a biological phenotype has not been found.

Seven variants of PSV–satellite RNA have been characterized, all of them associated with PSV isolates of the eastern type, six from the United States, and one from Poland (Collmer *et al*., 1985; Ferreiro

et al., 1996; Militao *et al*., 1998; Naidu *et al*., 1995). All of the PSV–satellite RNAs contain 393 nt with 99% similarity in sequence. They have a 5′-terminal cap and a 3′-terminal –CCC, and the sequences at both termini are homologous with those of CMV–satellite RNA; no other homologies with CMV–satellite RNA exist, but sequence similarities with the central conserved region of viroids and with introns have been reported (Collmer *et al*., 1985). PSV–satellite RNAs have potential open reading frames, although of unknown *in vivo* significance.

B. Replication

CMV–satellite RNA has been utilized as a model system for RNA replication. With one exception (Ix-CMV; Kaper *et al*., 1990), all CMV strains analyzed were able to maintain (i.e., replicate and encapsidate) different satellite RNA variants. The inability of Ix-CMV to support the replication of some satellite RNAs mapped to RNA 1 (McGarvey *et al*., 1995). The inability of a particular CMV strain to support satellite RNA in squash also mapped to RNA 1 (Roossinck *et al*., 1997). While all tested PSV isolates in the eastern group were able to support PSV–satellite RNA, some PSV isolates in the western group and the Robinia strain of PSV could not (Militao *et al*., 1998; Naidu *et al*., 1995). The inability of W–PSV to support PSV–satellite RNA mapped to RNA 1 (Hu *et al*., 1998). Hence, it appears that RNA 1 of cucumoviruses determines the maintenance of the satellite RNAs. Most, but not all, CMV–satellite RNAs are supported poorly in cucurbit hosts (García-Arenal and Palukaitis, 1999). The ability of a satellite RNA to accumulate to high levels in cucurbits did not map to any specific satellite RNA sequence, but appeared to be due to a higher-order structure (Bernal and Garcia-Arenal, 1994a).

Some TAV strains were able to support a variety of CMV–satellite RNAs (Gould *et al*., 1978; Harrison *et al*., 1987; Lee and Kummert, 1985). Other TAV strains did not support either the replication or the encapsidation and systemic movement of particular CMV–satellite RNAs (Jaegle *et al*., 1990; Moriones *et al*., 1992). TAV also showed preferential support of some satellite RNAs from mixtures (Roossinck and Palukaitis, 1995). Determinants in the satellite RNA and the helper virus for these properties have been analyzed (Bernal and García-Arenal, 1994b; Jaegle *et al*., 1990; Moriones *et al*., 1994; Roossinck and Palukaitis, 1995).

In plants infected by CMV plus satellite RNA, high levels of double-stranded satellite RNA accumulated (Diaz-Ruiz and Kaper, 1977). In contrast to helper virus RNA, the amount of (+) and (−) satellite

RNA that accumulates in infected tissues is of the same order of magnitude (Piazzolla *et al*., 1982). In addition to high levels of unit-length, double-stranded satellite RNA, multimers of both polarities also have been found in the tissues of infected plants, but circular forms have not been detected (Kuroda *et al*., 1997; Linthorst and Kaper, 1985). Multimers of both polarities, but not circular monomers, were also found for PSV–satellite RNA (Linthorst and Kaper, 1984). Hence, it is assumed that multimers are produced by the reinitiation of replication on the 3′ end before release of the nascent satellite RNA molecule (Kuroda *et al*., 1997). CMV-and PSV-satellite RNAs of (−) polarity have an additional guanosine at the 3′ terminus unpaired in double-stranded RNA (Collmer and Kaper, 1985). This has been proposed to play a role in multimer RNA synthesis and is required for (−) satellite RNA to be an efficient template for (+) strand satellite RNA synthesis (Wu and Kaper, 1994). The 3′ terminus of CMV–satellite RNA may be degraded and repaired *in vivo*, and deletions of up to seven nucleotides from the 3′ terminus were repaired *in planta* during replication by CMV (Burgyan and García-Arenal, 1998).

C. Pathology

The presence of a satellite RNA modifies the pathogenesis of CMV, and CMV–satellite RNAs have also been analyzed considerably in this respect. Depending on the strain of helper virus and satellite RNA and on the species of host plant, the effect of the satellite RNA on the pathogenicity may be none, an enhancement by the induction of necrosis or chlorosis, or an attenuation of the helper virus-induced symptoms. The last is by far the most common effect (García-Arenal and Palukaitis, 1999). The replication of CMV–satellite RNAs generally resulted in a reduction of CMV RNA accumulation (Habili and Kaper, 1981; Kaper, 1982). It had been suggested that the reduction in CMV accumulation in the presence of satellite RNA could be the cause of the satellite RNA-mediated attenuation of the helper virus-induced symptoms. However, CMV–satellite RNAs also attenuated TAV-induced symptoms, but did not reduce TAV accumulation (Harrison *et al*., 1987; Moriones *et al*., 1992), and satellite RNAs that induced necrosis in tomato plants also reduced CMV accumulation (e.g., Escriu *et al*., 2000a). Moreover, co-infection of CMV plus satellite RNA and the *Potyvirus Zucchini yellow mosaic virus* (ZYMV) in zucchini squash resulted in an increase in the levels of both CMV accumulation and satellite RNA accumulation (Wang *et al*., 2002). These data indicate that satellite RNA is not competing with CMV RNAs for limited amounts of CMV replicase.

Some CMV–satellite RNAs induce chlorosis in tomato or tobacco (but not in both species). A chlorosis-inducing domain has been mapped within the 5′ half of the molecule (Jaegle *et al*., 1990; Kurath and Palukaitis, 1989), and specific changes that affected the host specificity and the extent of chlorosis have been identified (Sleat and Palukaitis, 1992; Sleat *et al*., 1994; Zhang *et al*., 1994b). The induction of chlorosis depends on the presence of the helper virus, as expression of chlorosis-inducing satellite RNAs from transgenes or from PVX as a vector did not result in chlorosis induction (Masuta *et al*., 1989; Taliansky *et al*., 1998). Chlorosis induction in tobacco also depends on the strain of CMV and is controlled by RNA 2 (Sleat and Palukaitis, 1990). The genotype of the host plant also affected chlorosis induction where it was controlled by a single, incompletely dominant gene (Masuta *et al*., 1993).

The ability to induce a systemic necrosis in tomato plants depended on a domain that maps within the 3′ half of the satellite RNA (Devic *et al*., 1989; Kurath and Palukaitis, 1989; Masuta and Takanami, 1989), although sequences outside this domain influenced the extent of necrosis (Sleat *et al*., 1994; Wu and Kaper, 1992). Expression of a necrogenic satellite RNA from a trangene in tomato did not result in necrosis induction in the absence of CMV infection (McGarvey *et al*., 1990). However, expression of the (−) sense satellite RNA sequence, but not the (+) sense one, from a PVX vector resulted in tomato necrosis in the absence of CMV (Taliansky *et al*., 1998). Tomato cell necrosis induced by CMV–satellite RNA initiates in the phloem and cambium cells of developing internodes, is dependent on cell developmental processes, and correlates with a high accumulation of (−) sense satellite RNA at the outset. This cell necrosis has the characteristics of programmed cell death (Xu and Roossinck, 2000).

PSV–satellite RNA did not modify, or attenuate, the symptoms of PSV isolates in the eastern group, but aggravated the symptoms of PSV isolates in the western group. In no case was the accumulation of the helper virus affected, as occurred with CMV and CMV-satellite RNA (Ferreiro *et al*., 1996; Militao *et al*., 1998; Naidu *et al*., 1995).

D. Transmission and Population Dynamics

Cucumoviral satellite RNAs, encapsidated in the helper virus particles, are transmitted by aphid vectors. Because of the depression of CMV accumulation due to satellite RNA, the efficiency of transmission of isolates supporting a satellite RNA may be much less than for those without one (Escriu *et al*., 2000a; Jacquemond, 1982). Transmission of

satellite RNA to CMV progeny is highly efficient, but differences between CMV–satellite RNA variants have been reported (Escriu *et al*., 2000a,b).

It was apparent from early data that isolates of CMV–satellite RNA were heterogeneous mixtures of sequence variants (Richards *et al*., 1978) and that the prevailing sequence could change upon passage (Kurath and Palukaitis, 1990). Which particular sequence was selected for during passage experiments depended on several factors: the host plant species (García-Luque *et al*., 1984; Kaper *et al*., 1988; Kurath and Palukaitis, 1990; Moriones *et al*., 1991); the nature of the helper virus (Palukaitis and Roossinck, 1995; Roossinck and Palukaitis 1995); the environmental conditions (Kaper *et al*., 1995; White *et al*., 1995b); and the nature of the satellite RNA sequence itself (Palukaitis and Roossinck, 1995). However, in the field, no correlation has been found between satellite RNA variants and host plant or helper virus species (Alonso-Prados *et al*., 1998; Grieco *et al*., 1997), possibly because other factors, such as random genetic drift, overshadow any possible selection effect. In the field, CMV–satellite RNA evolution was determined primarily by mutation accumulation along major evolutionary lines corresponding to variants necrogenic or nonnecrogenic in tomato (Aranda *et al*., 1993; Grieco *et al*., 1997), although recombination between these lines also occurred (Aranda *et al*., 1997). It has also been shown that the population dynamics and genetics of CMV–satellite RNA and its helper virus are different and that satellite RNA spreads epidemically as a parasite on the population of CMV (Alonso-Prados *et al*., 1998). The ability of satellite RNAs to invade the population of satellite-free CMV is related to the effectiveness of transmission and is affected by the dynamics of the aphid vector population (Escriu *et al*., 2003). These observations, and those showing that attenuating satellite RNAs could evolve easily to exacerbating phenotypes (Palukaitis and Roossinck, 1996), indicate that some risk is associated with the widespread use of CMV–satellite RNAs for the biocontrol of CMV (Tien and Wu, 1991).

IX. Synergy with Other Viruses

Synergy is the interaction between two viruses resulting in a worse disease than caused by infection with either virus alone. In a synergistic interaction, there is usually an increase in the accumulation of one of the interacting viruses. Cucumoviruses have been shown to be involved in various forms of synergy. In some cases, such as in cowpea

stunt disease and in a severe mosaic disease of radish, CMV and a potyvirus [*Blackeye cowpea mosaic virus* (BICMV) and *Turnip mosaic virus* (TuMV), respectively] have been shown to be involved together in the synergy (Pio-Ribeiro *et al*., 1978; Sano and Kojima, 1989). In cowpea varieties showing no effect on the accumulation of BICMV but showing tolerance to disease symptoms, coinfection by CMV overcame the tolerance and also showed a synergy of CMV accumulation (Anderson *et al*., 1996). This indicates that the tolerance to BICMV had no effect on the ability of CMV to induce a disease response. However, in this system, coinfection by both viruses of a cowpea variety with extreme resistance to BICMV neither broke the resistance to BICMV nor resulted in any synergy of CMV accumulation or disease. In radish coinfected by TuMV and CMV, the synergy was shown to be due to TuMV enhancing the systemic movement and accumulation of CMV, but there was no change in the accumulation of TuMV (Takeshita and Takanami, 2000). Dual infection by *Sweet potato feathery mottle virus* and CMV was also shown to be required for infection of sweet potato (*Ipomoea setosa*) by CMV (Cohen *et al*., 1988). In contrast to the situation in radish and sweet potato, many other examples of synergy involve host plants in which both viruses can invade the plants efficiently.

A. Synergy in Solanaceous Plants

CMV was shown to interact synergistically with an uncharacterized virus from potatoes (probably PVX) in causing tomato streak disease (Valleau and Johnson, 1930), as well as with PVX (Ross, 1950) or TMV (Garces-Orejuela and Pound, 1957) in tobacco. Synergy of CMV in tobacco by several viruses has been shown to correlate with an increase in CMV accumulation (Palukaitis and Kaplan, 1997). Coinfection of tobacco by CMV and TMV, *Potato virus Y* (PVY), or PVX all stimulated CMV accumulation, whereas CMV only stimulated PVX accumulation and not the accumulation of either TMV or PVY (Palukaitis and Kaplan, 1997). Transgenic tobacco plants expressing the P1/HCPro region of the *Potyvirus Tobacco etch virus* (TEV) were able to enhance the accumulation and pathogenicity of CMV, although CMV satellite RNA replication was suppressed in this system (Pruss *et al*., 1997). This suggests that the P1/HCPro is responsible for synergistic interactions of potyviruses with CMV. Although TMV also interacts synergistically with CMV, transgenic tobacco plants expressing different segments of the TMV genome (encoding the 126-kDa protein, the 54-kDa protein, the 30-kDa protein, or the capsid protein) did not

interact synergistically with CMV (Anderson *et al*., 1989; Palukaitis and Kaplan, 1997). Thus, synergy between CMV and TMV may involve either more than one gene product of TMV or some intermediates associated with TMV replication.

CMV also interacts synergistically with a number of viruses, resulting in an enhancement of infection by those viruses. In pepper plants resistant to the *Potyvirus Pepper mottle virus* (PepMoV), coinfection by CMV broke the resistance against systemic infection by PepMoV (Murphy and Kyle, 1995). The upward movement of PepMoV in the internal phloem was blocked in the resistant pepper plants (Andrianifahanana *et al*., 1997), while coinfection by CMV overcame this resistance barrier, allowing this systemic movement of PepMoV in the internal phloem (Guerini and Murphy, 1999). Similarly, in *N. benthamiana*, double infection by the *Potyvirus Plum pox virus* (PPV) and CMV overcame the barrier to allow limited systemic movement of PPV (Sáenz *et al*., 2002). The breakage of resistance was not due merely to some function of the CMV 2b protein alone, as expression of the CMV 2b protein from PPV did not overcome the resistance barrier (Sáenz *et al*., 2002).

CMV was also able to overcome resistance mediated by transgenes. The resistance in tobacco plants showing RNA-mediated resistance to PVY could be overcome by either coinoculation or prior inoculation with CMV (Mitter *et al*., 2001; H. Barker, personal com.). Resistance breakage was shown to be associated with alleviation of gene silencing of the viral trangene (Mitter *et al*., 2001) and the ability of CMV to overcome the resistance-required expression of the 2b gene (H. Barker and M. Taliansky, personal com.).

Umbraviruses can facilitate the mechanical infection of luteoviruses (Mayo *et al*., 2000). While CMV cannot do so, CMV expressing the GRV 28-kDa movement protein in place of the CMV 3a protein could facilitate mechanical infection of the *Potato leafroll virus* in *N. benthamiana* (Ryabov *et al*., 2001). Such movement was again conditional on the presence of the 2b gene in the hybrid CMV/GRV genome.

B. Synergy in Cucurbitaceous Plants

Curcurbit plants in the field have exhibited synergy of virus infection involving CMV (Grafton-Cardwell *et al*., 1996; Luis-Arteaga *et al*., 1998). Synergy between the potyvirus ZYMV and CMV was observed in cucumber (Poolpol and Inouye, 1986), melon, and zucchini squash (Wang *et al*., 2002). In all three hosts, synergy in pathogenicity was correlated with an increase in the level of CMV accumulation, with

little change in the level of ZYMV accumulation. Infection of ZYMV plus CMV containing a satellite RNA did not suppress satellite RNA accumulation (Wang *et al*., 2002), as in transgenic tobacco expressing the TEV P1/HCPro infected by CMV containing satellite RNA (Pruss *et al*., 1997). The reasons for these differences in effects on satellite RNA accumulation are unknown. Synergy of pathogenicity in zucchini squash after infection with the *Potyvirus Watermelon mosaic virus* and CMV resulted in similar increases in CMV accumulation. In none of the aforementioned plants was there an increase in the level of the (−) RNAs of CMV, but only of (+) CMV RNAs and capsid protein (Wang *et al*., 2002).

Lines of cucumber tolerant to CMV infection showed little accumulation of CMV. However, coinfection of such plants by CMV and ZYMV resulted in synergy of infection, with breakage of the resistance to CMV accumulation. An attenuated strain of ZYMV also showed the same synergistic effect with CMV in CMV-tolerant cucumber plants, and the attenuated ZYMV expressing the 2b gene of CMV induced hypervirulence on cucumber and melon. Thus, ZYMV could overcome resistance to virus accumulation manifested against CMV, and the CMV 2b gene could overcome a tolerance phenotype conferred by the attenuated strain of ZYMV (Y. Wang, P. Palukaitis, and A. Gal-On, unpublished data). The aforementioned resistance against CMV also inhibited accumulation of CMV in protoplasts from the resistant cultivar, and ZYMV stimulated CMV RNA accumulation in these protoplasts, but only to a slight extent in protoplasts from a susceptible cultivar (K. C. Lee, S.-M. Wong, A. Gal-On, and P. Palukaitis, unpublished data). This is consistent with earlier conclusions that increases of CMV accumulation in a susceptible cucumber cultivar, as a function of a synergistic interaction with ZYMV, were due to an increase in the number of cells infected, rather than an increase in the level of CMV per infected cell (Poolpol and Inouye, 1986). In contrast, the resistance breakage mediated by ZYMV, which allowed for an increase in CMV accumulation in single cells, appears to operate by a mechanism distinct from that allowing further cell-to-cell and long-distance movement.

The resistance response in zucchini squash specifically preventing the M strain of CMV from infecting this host systemically (Wong *et al*., 1999) could also be overcome by coinfection of M–CMV with ZYMV (Choi *et al*., 2002). The level of accumulation in systemic leaves of this host by M–CMV in the presence of ZYMV was similar to that of Fny–CMV alone, which is not restricted in the same host (Choi *et al*., 2002). Again, this indicates that synergy of accumulation did not occur

in high levels when the synergistic interaction occurred in a plant species with a defined resistance to one of the viruses. Thus, there appear to be limits on the extent of synergy that are defined by the number of host responses involved in limiting infection.

X. Future Prospects

A perusal of this review makes it quite apparent where the bulk of progress has been made in the last decade. Areas still in need of considerable research include, but are not limited to, the mechanisms of seed and aphid transmission, the nature of host components involved in various interactions leading to infection, resistance or pathogenicity, and the mechanisms of cell-to-cell movement and resistance breakage. New sources of resistance to virus infection, disease, or the vector will undoubtedly be discovered and characterized. If sufficiently broad in spectrum, some of these may see deployment in the field.

While it might have been expected that the nature of host components involved in replication and movement would have been determined already, the reality is that the low levels of these complexes in plants have not faciliatated rapid progress. In the case of some other virus–plant systems, a genetic approach using *Arabidopsis* ecotypes and mutants is being used to identify specific genes involved in interactions leading to susceptibility/resistance and pathogenicity. Several researchers have made progress using such screens toward isolating genes involved in cucumovirus–*Arabidopsis* interactions. It is hoped that more mutants will be identified that will allow plant genes involved in replication and movement to be isolated and characterized. However, it is not clear if resistance genes obtained from such screens can be utilized to engender resistance in the field. Many such resistance genes only appear to function in plants in the same taxonomic family, presumably due to the absence of other components in the resistance pathway. Moreover, previous experience has shown that resistance genes are often specific to only some strains of CMV. Thus, progress in engendering resistance based on the isolation and deployment of "natural" resistance genes against various cucumoviruses may take a considerable effort. However, there are various forms of pathogen-derived or transgenic, broad-spectrum resistance that are available, but in need of evaluation for efficacy in the field. Utilization of some of these is currently subject to moratoria in many countries, based on either biosafety or political concerns. The future use of transgenic resistance will require a better understanding of the evolution of

cucumoviruses and the factors that determine their evolution. Both the durability of the transgene-mediated resistance and its potential ecological risks will depend on the evolution of the virus being controlled. However, increases in global temperatures might result in changes in the distribution and fecundity of the aphid vectors of a number of viruses in temperate countries. This may lead to a reevaluation of the benefits versus the risks of the deployment of such resistance.

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ADVANCES IN VIRUS RESEARCH, VOL 62

COEVOLUTION OF VIRUSES WITH HOSTS AND VECTORS AND POSSIBLE PALEONTOLOGY

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The coevolution of viruses with their hosts and vectors depends on the evolution of the hosts and vectors coupled with factors involved in virus evolution. The long-term perspective involves the origin of life forms, the evolution of host and vector (especially arthropods) kingdoms and families, and changes in biological diversity induced mainly by the last five great extinctions. In the medium term, the diversification of hosts and vectors is important, and in the short term, recent events, especially humans, have had a great impact on virus coevolution. As there are few, if any, examples of conventional fossils of

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viruses, evidence for their evolution related to host and vector evolution is being found from other sources, especially virus-induced cellular structures and recent developments in molecular biology. Recognizing these other sources is becoming important for paleontologists gaining an understanding of the influence that viruses have had on the development of higher organisms.

I. Introduction

Paleontology plays a major role in the understanding of the evolution of most organisms. Even though no conventional fossils of viruses have been found, there are other approaches to viral paleontology that are attracting much interest. Using joint competencies (virology and paleontology), we aim to review various aspects of virus–host–vector coevolution in the frame of evolution of organisms and to make some suggestions for paleontologists working with well-preserved fossil organisms on how to detect evidence of ancient virus infections.

The knowledge we have of the host distribution of virus species in nature is greatly anthropocentric and related mainly to diseases of humans and of the animals and plants of interest for humans. Little is known on the virology of "natural" situations, and several topics, such as viruses of lower plants, viruses of nonarthropod invertebrates, and viruses of many wild vertebrate species, have been poorly investigated.

Analysis of the 3885 viruses described in Van Regenmortel *et al*. (2000) reveals that 59% are reported from vertebrates and insects, 25% from plants, 15% from bacteria, and less than 1% from fungi (Table I) (Hull, 2001). This contrasts with the worldwide percentage of known eukaryota species being 79% invertebrates, 18% plants, and 3% vertebrates (Clark and May, 2002).

Insecta and Acarina are arthropods that are very important in virology either because they are susceptible to the multiplication of many viruses or because they are propagative vectors of many vertebrate and plant virus diseases. The fact that arthropods are evolutionarily older than vertebrates and angiosperms suggests that they might have had a preeminent importance in the origin and evolution of several vertebrate and plant viruses.

To set the scene, we describe briefly the geological events important in the evolution of living organisms. A time line of geological era is shown in Fig. 1 together with major evolutionary events; details of evolutionary events relevant to this review are given in Table II. Among the most important factors in evolution that probably have the

TABLE I
VIRAL GENOMES IN HOST FROM DIFFERENT KINGDOMS[a]

Genome nucleic acid[b]	Plants		Fungi		Animal		Bacteria	
	No.	%	No.	%	No.	%	No.	%
dsDNA	0	0	1	3.5	606	26.5	445	75.4
ssDNA	166	17.0	0	0	58	2.5	88	14.9
RT	31	3.2	0	0	112	4.9	0	0
dsRNA	45	4.6	27	93.0	383	16.7	1	0.2
(−)ssRNA	100	10.2	0	0	604	26.5	0	0
(+)ssRNA	635	65.0	1	3.5	525	22.9	57	9.6
Total	977		29		2288		591	

[a] From Hull (2001), with permission.
[b] ds, double stranded; ss, single stranded; RT, viruses that replicate by reverse transcription—they may have either DNA or RNA genomes.

greatest impact on the coevolution of viruses with their hosts and vectors are the last five great extinctions, the splitting of the supercontinents, and humans. The following is a summary of the impact that extinctions had on potential virus hosts, the flora, and fauna.

As can be seen from Fig. 1 and Table II, there were five great extinction events since 435 million years ago (mya): the end of the Ordovician, the Devonian, the end of the Permian, the late Triassic, and at the Cretaceous–Tertiary boundary. At each of these events, there were major readjustments of the fauna and flora.

The five extinctions had considerable impacts on the detailed relationships between animals and plants. However, it is likely that the basic relationships leading to possible coevolution of virus–host associations became particularly important after animals and plants moved from water to land.

As well as cataclysmic events that changed the fauna and flora, the movement of landmasses is likely to have had a major impact on the coevolution of viruses and their hosts. For virus spread there has to be contact, either direct or indirect via a vector, between hosts. The movement of landmasses has separated and brought together evolving communities of such hosts. The recent understanding of continental drift is that in the pre-Pangea time land masses converged to make the supercontinent, Pangea, at around the Carboniferous (about 300 mya). Pangea started to split up in the Triassic (200 mya) to give Laurasia in the north and Gondwana in the south. These further split

TABLE II

PALEONTOLOGY OF PHYLA POSSIBLY INVOLVED IN VIRUS EVOLUTION

Eons, eras, and systems	Millions years ago	Paleontological events	Possible steps of virus evolution
Cenozoic			
Quaternary	0.010	Development of agriculture	Short-term coevolution of viruses
	0.018–0.011	Multiple Pleistocene glaciations	Extinction of some groups of viruses
Neogene	2	First Hominidae	First evolution of human viruses
	23	Disappearance of big reptiles; burst of insects, angiosperms, and conifers	Extinction of some reptile viruses; further evolution of plant and insect viruses
Paleogene	40	Important evolution of mammals; appearance of primates	Evolution of mammal viruses
	53	Global warming at the Paleocene/Eocene boundary; increase of herbivorous insects activity	Increase of relationships of viruses with insects and plants
	55–57	Spread of mammalian groups from Asia to Europe and North America	Introduction of mammal viruses to new continents
	64–65	Beginning of the explosive radiation of Ornithurae subclass of birds	Evolution of bird viruses
Mesozoic			
Cretaceous	65	Cretaceous–Paleogene fifth extinction: massive bird (subclass Sauriurae) and total dinosaur extinction	Loss of viruses in extinct vertebrates, invertebrates, and seed plants in North America
	65–95	Adaptation of acari to plants	Relationships of plant and acari viruses
	100–120	Opening of South Atlantic ocean. First placental mammals radiation	Geographic localization of some virus species; passage of virus to mammals
	100–135	Burst of Angiosperms; formation of Amber with inclusions of organisms	Diversification and expansion of plant viruses

Jurassic	135–152	Earliest fossil angiosperm	Migration of viruses to angiosperms
	~150	Origin of precursor of birds (urvögels); presence of Acarina eggs on a bird feather	Passage of viruses from reptiles to birds through blood-sucking parasites
	160	Beginning of pollination by insects	First evolution of pollen-transmitted viruses
	170	Divergence of marsupials	
	180	Evolution of Homoptera and Coleoptera; presence of fossil Gymnosperm pollen; beginning continental drift	Relationships of plants, insects, and viruses; localization of some viruses
	200	Fourth great extinction (reduction of marine fauna and of Pteridophytes)	Extinction of some virus groups
	~200	Flourishing of dinosaurs; origin of mammaliaforms	Beginning of evolution of viruses in mammals
Triassic	225	First fossils of Diptera	Relationships of Diptera and vertebrate viruses
	230	Evolution of Longidoridae nematodes	Relationships of nematodes with plants and viruses
		Evolution of mammal-like reptiles	
Paleozoic	250	Earliest fossils of Cycadales	Diversification of plant viruses
Permian	250–280	Big development of Algae and Pteridophyta; Glossopterids predominance in Gondwana	Further diversification of plant viruses.
	251	Permian–Triassic third great extinction (many species of plant and invertebrates); changes in insect diversity; replacement of primeval vegetation by a Gymnospermae dominated one	Extinction of some virus groups; evolution of insect and plant viruses
	280–295	Burst of insect development; appearance of wings, first herbivorous insects	Increasing relationship of plant and insect viruses
Carboniferous	~310–355	Evolution of seed plants (primitive Gymnospermae)	Evolution of plant viruses
	315–364	Burst of Fusulinidae (Protozoa).	Diversification of viruses in Protozoa
	320–330	Appearance of Blattaria and Orthoptera	Relationships of biting insects and viruses

(*continues*)

TABLE II (*continued*)

Eons, eras, and systems	Millions years ago	Paleontological events	Possible steps of virus evolution
	320–420	Evolution of Pteridophyta that spread over large forests	Diversification of plant viruses
	345	Appearance of Reptilia	Further diversification of poikilotherm vertebrate viruses
Devonian	355–405	First wingless insects (Collembola); evolution of fungi as saprophytes, parasites and mutualists; association of plants with detritus feeding arthropods	Relationships of prokaryote and eukaryota viruses through arthropods and fungi
	370	Second great extinction (marine families).	Extinction of some virus groups
	395	First amphibians	Evolution of poikilotherm viruses
Silurian	420	First great extinction of marine invertebrates; dawn of vascular plants and of animal life on land	Extinction of some virus groups; first diversification of animal viruses on land
Ordovician	440–450	First Pteridophytes, arachnids, and centipedes on land	First diversification of plant and arthropods viruses on land
	460–500	First record of Bryophytes and presence of Glomales mycorrhizal fungi	First connections of viruses with fungi and plants
	480–500	First known marine vertebrates (Agnatha, Heterostraci)	Diversification of animal viruses
	500	Explosion of animals to form most of the major extant phyla	Evolution of animal viruses

Cambrian	520–550	Origin of Chordates (mainly fishes)	Diversification of animal viruses
Proterozoic	580	Proliferation of first multicellular animals	Diversification of animal virus infections in algae and hydrozoan symbionts
	600	Varanger glaciation; first algae mass extinction; split of Ascomycete and Basidiomicete	Extinction of some virus groups; transfer and differentiation of viruses
	600–800	First multicellular algae, differentiation of green, red, brown algae and fungus-like organisms.	Connection and diversification of viruses in algae, fungi, and multicellular animals
	~670	Deuterostome and protostome split	Diversification of animal viruses
	900–1000	Fungus and multicellular animal split	Diversification of viruses
	1000–1600	Nutritional hard time for ocean life	
	~1230–1260	Origin of plastid in plants	
	~1230–1260	Protist split from plants, fungi, and animals	First evolution of protist viruses
	1600	Borderline between the plant and the animal kingdoms	First separation of plant and protozoa viruses
	1700–1900	Oldest fossils of eukaryotes	
	1800	First planktonic algae in geological records	Evolution of viruses in algae
	2000–2700	Prokaryote–eukaryote split	Migration of viruses in eukaryotes
Archaean	2500–2700	Cyanobacterial biomarkers and microfossil records	Evolution of prokaryote viruses
	~2800	Origin of photosynthetic bacteria	Relationships of viruses with photosynthetic organisms
	3500	Origin of prokaryotes	Origin of viruses in prokaryote cells
	~3800	Precellular life forms	Precursor of viruses in the early RNA world

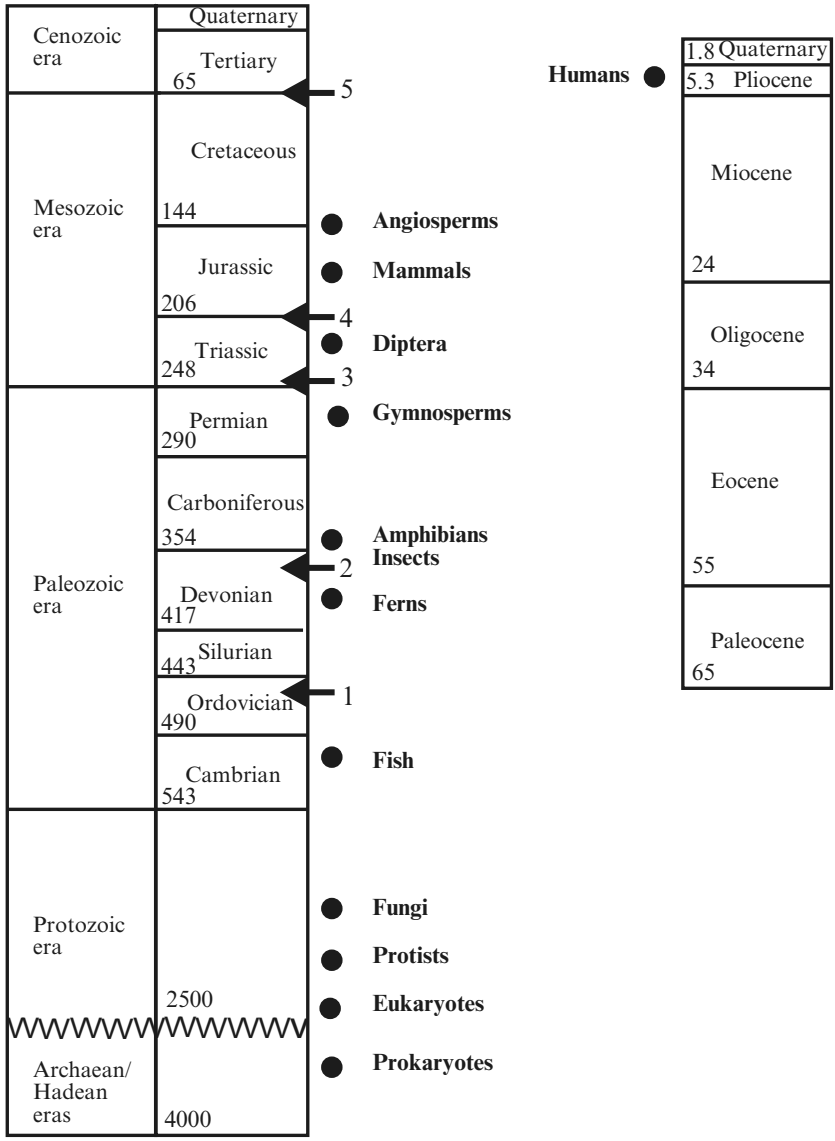

FIG 1. Chronogram of geological era and events important in the coevolution of viruses, their hosts, and vectors. Arrows indicate the five major extinctions and dots the first stages of the evolution of the indicated organisms. The numbers are mya. Detailed events are given in Table II.

during the late Cretaceous (100 mya), resulting in the basic current land mass distribution. However, it must be remembered that landmasses converged as well as split; most of the Indian subcontinent is a fragment of Africa that migrated as a crustal plate impacting on south Asia.

The third major factor in the evolution of both viruses and their hosts is the influence of humans. This is discussed in detail in Section V.

Over the last two decades, several papers on virus evolution have been published, and the main aspects of virus evolution have been treated in two monographic books edited by Gibbs *et al*. (1995) and by Domingo *et al*. (1999a) and in the Congress on "The Origin and Evolution of Viruses" (see Balter, 2000). Previous papers on evolution and possible paleontology of viruses, with emphasis on those of plants and insects, were published by Lovisolo and Boccardo (1996) and Lovisolo and Rösler (2001, 2003). Concise data on virus evolution and most of the information on virus taxonomy were taken from Granoff and Webster (1999), Hull (2001), and van Regenmortel *et al*. (2000).

Coevolution of viruses, vectors, and hosts can be considered in three time dimensions: long term relating to the origin of life forms and viruses and the evolution of host and vector kingdoms and families; medium term in the diversification of viruses; and short term in the impacts that recent events, especially humans, have had on coevolution. This review attempts to analyze these three time dimensions to bring together the factors leading to the current situation.

II. Paleontology of Hosts Involved in Virus Evolution

This section discusses some main paleontological events relevant to hosts and vectors of viruses that are considered important for their coevolutionary relationships.

Two important events that concern all eukaryotes are the origin of mitochondria and of chloroplasts. Meyerowitz (2002) noted that the uptake of alpha proteobacterium that led to mitochondria occurred before the separation of plants and animals (1600 mya). After this separation, the other important endosymbiotic event was the uptake of a cyanobacterium to form the precursor of chloroplasts, which occurred only in the plant lineage. Yoon *et al*. (2002) suggested that chromist algae share a common plastid that most likely arose from a single, ancient (~1260 mya) secondary endosymbiosis involving a red alga. The first cyanobacterial microfossil records date 2500 mya (see Des Marais, 2000).

A. Early "RNA World" and Prokaryotes

There are many theories on the development of living forms from nonliving ones (see Schopf, 2002), but there is strong evidence that the first self-replicating macromolecules were RNA rather than DNA (see Joyce, 2002). However, the precursor of possible early viruses and viroids needed cell systems for their replication, and these systems were probably Archaea and Bacteria prokaryotes.

Permanent whole cell fusion between members of Archaea (e.g., *Thermoplasma*-like organisms) and of Eubacteria (e.g., *Spirochaeta*-like organisms) is suggested to have given rise to Eukarya (Margulis, 1996). If viruses originated in bacteria, this should have favored their migration into the new Eukarya phyla. Horizontal gene transfer should have been an important factor in the evolution of viruses, as reviewed in Arber (1999) and Domingo *et al*. (1999a).

Microfossils, stromatolites, and biomarker compounds indicate that microbial organisms inhabited the oceans in Achaean times (Brocks *et al*., 1999; Knoll, 1999). Fossil cyanobacteria have been found in Canadian cherts of 2000 mya (Knoll, 1992), and the major photosynthetic groups of bacteria arose before 2800 mya (Des Marais, 2000).

B. Plants

1. Algae

Algae are the oldest eukaryota present in the Proterozoic aeon (Amber and Knoll, 2002), rhodophytes in the anoxic and sulfidic oceanic conditions of the Mesoproterozoic era, and chlorophytes at the beginning of the oxic conditions.

The oldest fossils of eukaryotes are from strata laid down between 1700 and 1900 mya (Knoll, 1992). Analysis of protein sequences indicated that green algae and major lineages of fungi were present at least 1000 mya (Heckman *et al*., 2001).

2. Bryophytes and Pteridophytes

An event important for the evolution of plant viruses could have been the origin of terrestrial plants, especially vascular ones with expanded leaves and flowers. Bryophytes were among the first land plants evolving from green algae in the Ordovician (ca. 500 mya). The first vascular plants (Rhyniophyta) evolved at the end of the Silurian (ca. 415 mya). Pteridophytes evolved in the Devonian (ca. 400 mya) and became the dominant vegetation. Viruses have never been

reported from bryophytes and have been rarely found in pteridophytes. A tobra-like virus was found in *Phyllites scolopendrum* (Hull, 1968).

3. Gymnosperms

After the Permo-Triassic extinction (250 mya), there was the replacement of primeval pteridophytes by gymnosperms (Anderson *et al*., 1999). In that period, plant viruses could have been present mainly in the root systems with possible relationships with soil nematodes, protozoa, and fungi. In the late Carboniferous, the appearance of Cordaitales, which had phloem near the stem surface, may have been a key process, leading to the widespread evolution of plant-feeding Hemiptera (Purcell, 1982).

Few viruses have been reported from Gymnospermae and these are frequently latent (Enunlu *et al*., 2003; Flachmann *et al*., 1990; Nienhaus and Castello 1989). Other plant viruses have been detected in soils, conifer roots, and percolating waters from forestry systems (Büttner and Nienhaus 1988). Interestingly, four different soil-borne viruses have been detected in the roots of conifers, but not in the leaves (Cooper 1993).

The only virus found to be pathogenic in extant Cycadales is the nepovirus *Cycas necrotic stunt virus*, isolated from *Cycas revoluta* (Hanada *et al*. 1986). Fossils of Cycadales are known from more than 250 mya.

4. Angiosperms

The earliest fossil angiosperm is the *Archaefructus liaoningensis* discovered from the Upper Jurassic layers of volcanic rocks sandwiched between sedimentary rocks of northeast China (Sun *et al*., 1998). Angiospermae diversified mainly during the Cretaceous and the most modern families had evolved by 60–80 mya (Raven, 1983).

Pollination of plants by insects began in the Upper Carboniferous period (Crepet, 1983) and became widespread during the Cretaceous. The diffusion of plant virus diseases through pollen could have started in those periods.

Few viruses are known for the extant angiosperms of ancient origin, such as Amborellaceae, Nymphaceae, Illiaceae, Magnoliaceae, and Lauraceae (see Sun *et al*., 2002).

C. Protists

It is impossible to discuss a Protozoan taxon without acknowledging that it is paraphyletic or even polyphyletic. Both animals and plants had their origin from a heterogeneous assemblage of mostly unicellular

organisms. Furthermore, it is sometimes difficult to say what is a flagellated "fungus" and what is a flagellated "protist" (Minelli, 1993).

With a protein clock, Doolittle *et al*. (1996) calculated that the protists split from the line leading to plants, fungi, and animals 1228–1236 mya.

D. Fungi

It is generally accepted that fungi do not have a monophyletic status (see Minelli, 1993) and that Plasmodiophorales are considered protozoa rather than fungi (Castlebury and Domier, 1998). Fungi originated in the Proterozoic (Blackwell, 2000; Doolittle *et al*., 1996; Redecker *et al*., 2000). The split between animals and fungi happened 900–1000 mya, whereas the Ascomycetes–Basidiomycetes split was 600 mya (Table II). The Glomales (Zygomycetes) probably first appeared in the Proterozoic. Molecular evidence obtained with protein sequence analyses indicates that green algae and a major lineage of fungi were present 1000 mya (Heckman *et al*., 2001).

Fossilized hyphae and spores from Ordovician (about 460 mya) strongly resemble modern arbuscular mycorrhizal Glomales. These fossils were present when the land flora probably only consisted of bryophytes, and these fungi may have played a crucial role in facilitating the colonization of land by plants (Redecker *et al*., 2000).

There is a wide range of associations between fungi and other organisms, mainly plants and animals, and coevolution is considered a major factor in their development (Pirozynski and Hawksworth, 1988).

E. Animals

Doolittle *et al*. (1996) used amino acid sequence data from 57 enzymes to determine the time of divergence of the major biological groups of living organisms. He suggested that deuterostomes (echinoderms, hemichordates, urochordates, and chordates) and protostomes (arthropods, nematodes, annelids, and molluscs) separated about 670 mya. Plants, animals, and fungi shared a common ancestor about a billion years ago.

1. Invertebrates

a. Arthropods As noted in Section II,B,4, arthropods started an association with plants (pollination) in the Carboniferous. The earliest phytophagous Coleoptera (Curculionoidea and Chrysomeloidea)

originated in the Triassic (248–206 mya) (Grimaldi, 2000). This was considerably earlier than the association of insects with vertebrates.

Various insect orders are of great importance in their vectoring plant viruses or being infected with viruses. Among the earliest of these to evolve were the Aleyrodidae originating in the Early Cretaceous (120–140 mya), Aphididae and Tingidae in the Berriasian age of the Early Cretaceous (128–135 mya), and Miridae (Heteroptera) in the Oxfordian age of the Jurassic (146–154 mya); the oldest Forficulidae are of the Hettangian age of the Early Jurassic (200 to 203 mya) and Acrididae (Orthoptera) in the Carnian age of the Late Triassic (220 to 230 mya) (see Ross and Jarzembowski, 1993). Earliest fossils of Diptera date 225 mya (Blackwell, 2000). Pseudococcidae originated in the Priabonian age (33.7–37 mya), and Membracidae originated in the Rupelian age (28–33.7 mya) (see Ross and Jarzembowski, 1993). The oldest Thripidae (Thysanoptera), vectors of circulative–propagative viruses, are from the Cenomian age of the Late Cretaceous (92–96 mya). The adaptation of acari (phytophagous mites) to plants started during the Late Cretaceous (Table II; Krantz and Lindquist 1979).

Arthropods could have been important also for the evolution of animal viruses. Vertebrate blood feeding, at least by extant ectoparasitic insect groups, is relatively recent in the history of tetrapods (Grimaldi, 2000). Culicidae Diptera originated in the Danian age (59–65 mya) of the Cenozoic era, and Psychodidae (to which belong the sandflies vectors) in the Aalenian age (176–180 mya) of the Jurassic (see Ross and Jarzembowski, 1993). Argasidae ticks, which feed on vertebrates, were found in Cretaceous amber (Klompen and Grimaldi, 2001).

b. Nematodes Logidoridae probably derive from an ancestor during the Devonian or Carboniferous. The genus *Xiphinema* would appear to be the oldest form and likely originated in Gondwana during the Permian when the flora was mainly seed ferns (*Glossopteridales*). The genus spread to Laurasia before the Pangea was torn apart, but the main speciation occurred in Africa. The center of origin of the genus *Longidorus* appears to have been southeast Asia and India (Brown and Trudgill, 1989).

c. Other Invertebrates Molluscs and tunicates evolved during the Cambrian and diversified during the Ordovician. Various phyla of them survived the five extinctions. However, few viruses are known to infect them. A lethal and common disease of octopus caused by an irido-like virus has been described by Rungger and colleagues

(in Gibbs, 1973). Virus-like particles of the enterovirus type were found in the tunicate *Pyurus stoloniferum* (Gibbs, 1973). Herpesviruses are known to infect oysters (see Nahmias, 1974), but their main hosts are vertebrates, mainly humans. For the ecology and epidemiology of several *Caliciviridae*, bivalve molluscan shellfish are very important. According to Smith (2000), a large number of infectious caliciviruses can be scavenged from the water column by molluscan shellfish and then retained for long periods.

2. Vertebrates

Hedges and Kumar (2002) compared vertebrate genomes on the basis of haploid genome mass and made an evolutionary tree indicating the beginning of the evolution of marsupials during the Jurassic and that of other terrestrial mammals clades during the lower Cretaceous (Table II). These data are in line with those of Murphy *et al*. (2001) on molecular phylogenetic studies on early placental mammal radiation. However, the finding of a new mammaliaform from the Early Jurassic (~195 mya) (Luo *et al*., 2001) is leading to a reevaluation of mammalian evolution.

Bowen *et al*. (2002) and Beard (2002) consider Asia as a center for early mammalian origin that later (55–57 mya) spread to Europe and North America. The Cenozoic era, generally considered the age of mammals, is when the first important evolution and diversification of viruses of mammals probably happened, favored by the fact that at least the extant arthropod vectors of animal viruses were present (see Section II,E,1,a).

The main diversification of viruses of mammals was probably more recent because mammals had important evolution in the Paleogene period of the Cenozoic era. During this period, relationships among plants, mammals, and arthropods could have been more close than they are today and some groups of viruses could have evolved in the three phyla from the same ancestral viruses (Lovisolo and Rosler, 2001).

In comparison with the knowledge on viruses of warm-blooded animals, relatively little is known about viruses infecting cold-blooded vertebrates (Chinchar, 2000). This in contrast with the fact that there are about 4500 bird species, 30,000 bony fish species, 4000 amphibian species, and 7000 reptile species but only 4500 mammalian species.

Birds originating in the early Cretaceous (~144 mya) most probably derived from the primitive reptilian birds of the Late Jurassic (150 mya). The subclass Sauriurae of land birds were dominant in the Mesozoic, and endured extinction at the Cretaceous–Tertiary (K-T)

bottleneck (Feduccia, 1995). After K-T extinction, birds of the Ornithurae subclass began a modern, explosive adaptive radiation.

III. Virus Origins and Evolution

As noted earlier, the lack of conventional fossil evidence has rendered impossible the usual approaches to understanding virus evolution. However, there is increasing evidence in this area from other sources, which are discussed in this section.

A. Virus Origins

Molecular studies of viruses are shedding light on a possible common origin of some animal and plant viruses. There are numerous suggestions as to the origins of viruses that essentially fall under three headings: descent from primitive precellular life forms, such as RNA, that later parasitized the earliest cells; development from normal cell constituents (e.g., transposable elements and mRNAs) that become self-replicating entities; and derivation from degenerate cells that eventually parasitized normal cells (see Hull, 2001; Schuster and Stadler, 1999). However, it has also been suggested that the eukaryotic nucleus derived from a complex DNA virus (Bell, 2001).

There is accumulating evidence for the modular or "cassette" hypothesis in the evolution of viruses and for their adaptation to specific hosts. This hypothesis, originally proposed by Botstein (1980) for DNA bacteriophage, suggests that viruses have evolved by recombinational rearrangements or reassortments of interchangeable elements or cassettes. Cassettes are defined as interchangeable genetic elements, each of which carries out a distinct biological function, such as replication or encapsidation of the viral genome. The hypothesis has been extended further to suggest that viral adaptation to the type of host (animal, plant) is mediated by the acquisition of host-specific cassettes (Hull, 1992, 2001; Hull and Covey, 1996).

The question arises as to where the basic replicon(s), comprising the polymerase and other gene products and sequences that are essential for replication, came from. There are three basic replicon types: (a) transcribing RNA from RNA using RNA-dependent RNA polymerase (RdRp); (b) transcribing DNA directly from DNA using DNA-dependent DNA polymerase (DdDp); and (c) transcribing between DNA and RNA (reverse-transcribing viruses) using DNA-dependent RNA polymerase (DdRp) and RNA-dependent DNA polymerase

(reverse transcriptase, RT). Structural studies have shown considerable similarities among RdRp, DdDp, DdRp, and RT (Hansen *et al*., 1997; Hingorani and O'Donnell, 2000; Kohlstaedt *et al*., 1992). If the "RNA world" suggestion of the origin of life forms is acceptable (Section II,A), some form of RdRp would likely to have been the ancestral polymerase. RT would be needed to convert the ancestral RNA to DNA, and DdDp and DdRp would be needed for the replication and expression of cellular DNA. The basic replicon of reverse-transcribing viruses contains RT and these viruses depend on host DdRp for their replication.

One exception to the aforementioned basic replicon hypothesis is viruses with single-stranded DNA genomes, which use the host DdDp. The host polymerase is easily available for single-stranded (ss) DNA viruses of prokaryotes (*Inoviridae* and *Microviridae*), the replicon "cassette" of the virus providing functions that assist replication. However, most of the cells infected by such viruses of eukaryotes (*Geminiviridae, Circoviridae, Parvoviridae*, and *Nanovirus*) are in the stationary (G) phase and have shut down most of their DNA replication activity. The replicon "cassettes" of these viruses contain genes, the products of which convert the infected cells from the G phase to the DNA replication-active S phase (reviewed in Gutierrez, 1999; Hanley-Bowdoin *et al*., 1999).

B. Factors Affecting Virus Evolution

The evolution of viruses, as with the evolution of other organisms, depends on variation and selection pressures on that variation. There are three major sources of variation in viruses: mutation, pseudorecombination, and recombination.

The rapid replication of viruses and the lack of proofreading and repair mechanisms by the polymerases, RdRp, DdRp, and RT give the potential of much variation, especially in RNA and reverse-transcribing viruses; DdDps usually have proofreading mechanisms and thus DNA viruses using this polymerase have less propensity for such variation. Variation leads to viruses being "quasispecies" with each virus population comprising one or more "master" sequence(s) and a very large number (a "cloud") of variants. Changes in environment act on this cloud of quasispecies and can give rise to genetic drift in which a new "master" sequence is selected. For a detailed discussion of quasispecies, see Domingo *et al*. (1999b) and Smith *et al*. (1997).

Pseudorecombination (also termed reassortment) can take place in those viruses that have their genome divided between several nucleic

acid fragments (e.g., *Orthomyxoviridae*, many plant viruses). In joint infections between different strains, equivalent genome fragments can be exchanged, giving a combination of properties of the two parent strains.

Recombination occurs both within RNA and DNA. Recombination can lead to direct exchange between equivalent parts of a viral genome, to deletions or additions within a viral genome, or to rearrangement of the viral genome (reviewed for plant viruses by Hull, 2001). Successful recombinations can lead to large changes in a virus and are likely to be responsible for major steps in viral evolution.

Selection pressures are related to the ability of a virus to coexist with its host and to the mode of existence of the virus itself. These reflect driving forces for the coevolution of viruses, their hosts, and their vectors.

A successful virus needs to maintain its host in a living state for as long as possible. Thus, it needs to maintain a balance between being able to replicate to a sufficient level to be transmitted to another host without compromising its original host. It also has to overcome host resistance mechanisms such as restriction enzyme systems in prokaryotes, immune systems in vertebrates and RNA silencing in plants, fungi, and certain animals. There is a wide variety of ways in which resistance mechanisms are overcome, including modification to stop restriction endonucleases, variation or avoidance to overcome immune systems, and suppressors to overcome RNA silencing (Baulcombe, 2002).

Selection pressures directly attributable to the virus include the ability to be able to move efficiently from an infected to a healthy host and the efficiency at all stages of the replication cycle. As noted earlier, transmission efficiency has to be balanced against the impact the virus infection has on the host. Efficiency in the replication cycle is reflected in genome organization with the genes being switched on and off at the appropriate time and in the appropriate place. It is also associated with genes and their products having multiple functions. For example, the coat protein of the geminivirus *maize streak virus* protects the viral genome, associates with the leafhopper vector, and provides a nuclear shuttle function in viral replication (see Hull, 2001).

C. Virus Evolution in Relation to Host Evolution

Several relevant issues are raised from the discussion of host evolution in Section II.

1. Gorbalenya (1995) suggested that ancestral viruses were present before the separation of prokaryotic and eukaryotic lineages (2700 and

2000 mya) (see Doolittle *et al*., 1996; Meyerowitz, 2002; Morell, 1996). Virus evolution probably started in earlier prokaryotes. Because several different type of viruses, mainly complex DNA ones, are present in Archaea and Bacteria, we may suppose that these organisms have been important for the origin and early evolution of viruses. There is an indication for the existence of head and tail viruses before the divergence of Archaea and Bacteria that may support the hypothesis that head and tail phages are derived from a common viral ancestor, already existing before the divergence of the three domains of life (Arnold *et al*., 1999).

2. The discovery that *Buchnera* bacteria, endosymbionts of aphids and whiteflies, are necessary for the circulative transmission of luteoviruses and geminiviruses through a GroEL chaperonin protein (Czosnek *et al*., 2001; Van Den Heuvel *et al*., 1997) is an indication that some prokaryotes may have had an important role in the evolution of viruses. Virus-transmitting insects and their endosymbionts have interacted from at least 200–100 mya (Baumann *et al*., 1995; Czosnek *et al*. 2001).

3. The evolution of arthropods, especially insects (which became the largest phylum in the animal kingdom, containing about the 75% of all animal species), and their relationships with both terrestrial plants and vertebrates were probably important for the diversification of viruses. That could have started during the Late Paleozoic, but should have expanded rapidly during the Mesozoic with evolution of the angiosperms and their even more important relationships with arthropods, fungi, and prokaryotes.

4. Gibbs *et al*. (1999) suggested that viruses of the Alpha-like virus group (ALVG) and charophycean algae had a very long-term association as these algae are the probable ancestors of land plants (Kranz *et al*., 1995): the earliest charalean fossils are dated as the Late Silurian–Devonian period (420 mya) (Grant, 1989; Tappan, 1980). *Chara australis* virus (CAV), a tobamo-like virus, isolated from *Chara australis*, a Charophycea alga (Skotnicki *et al*., 1976), resembles morphologically *tobacco mosaic virus* (TMV), but its genome is bigger. Süss *et al*. (1965) demonstrated the replication of infective TMV RNA in *Chlorella pyrenoidosa*, but CAV is the only virus related to flowering plant viruses found in algae in nature.

5. The root of tobamoviruses phylogeny may correspond to prototobamoviruses infecting the earliest angiosperms. Major tobamovirus radiations, resulting in the clusters now found in the Solanaceae, the legumes and cucurbits, may have occurred when these modern families radiated 60–80 mya (Gibbs, 1999). This period included both the

final stage of dismemberment of Gondwana and the Cretaceous–Tertiary extinction boundary (Schultz and Dhondt 1996), events that may also account for the deep branches within some of these tobamovirus groups.

6. The family *Phycodnaviridae*, with four genera, *Chlorovirus*, *Prasinovirus*, *Phaeovirus*, and *Prymnesiovirus*, contains important algal viruses (Van Etten, 1999; Van Etten and Meints, 1999). The first two genera have members that infect algal endosymbionts of protozoa (*Paramecium bursaria*) and cnidaria (*Hydra viridis*). Analysis of the DNA polymerase gene from selected members of the family *Phycodnaviridae* indicates that the viruses are more closely related to each other than to other double-stranded (ds) DNA virus and they form a distinct phyletic group, suggesting that they share a common ancestor (Van Etten, 2000). In addition, DNA polymerases of *Phycodnaviridae* share some relationships with those of *Baculoviridae, Poxviridae*, and *Herpesviridae*. Phycodnaviruses have not been reported in protist and animal symbionts. However, Chlorella virus genomes contain an interesting mosaic of prokaryotic- and eukaryotic-like genes (Van Etten, 1999), suggesting that these symbionts could have been a way for the algae to acquire viruses with affinities with animal viruses.

7. Most identified protozoan viruses belong to dsRNA *Totiviridae*. No infection phase has yet been recognized for most *Totiviridae* of fungi and protozoa but they appear to exist as permanent persistent infections, passing from cell to cell only by mating and cell division, which suggests that they originated prior to the differentiation of multicellular eukaryotes (Bruenn, 1999). Analysis of nucleotide sequences showed that some *Trichomonas vaginalis* and *Saccharomyces cerevisiae* viruses may have a common origin (Ghabrial and Patterson, 1999).

8. The relationships between Plasmodiophorales and plant viruses could be ancient. Particles of some viruses are carried internally in the zoospores and resting spores of Plasmodiophorales (Adams, 2002; Campbell, 1996). Gibbs (1999) hypothesized the possible involvement of Plasmodiophorales in the evolution of pretobamoviruses on the basis that although the mode of transmission of CAV is unknown, Plasmodiophorales are known to parasitize charalean algae and other species transmit furoviruses of the ALVG.

9. Gibbs and Weiller (1999) suggested that a plant ssDNA nanovirus switched to an animal host and then recombined with a vertebrate-infecting virus to become a circovirus. They speculate that the host–switch occurred when a vertebrate was exposed to sap from an infected plant.

10. Nucleotide sequence analysis of the envelope (E) gene revealed that yellow fever virus evolved early in the lineage of mosquito-borne flaviviruses, approximately 3000 years ago (Monath, 1999).

D. Evidence for Virus Evolution

The dearth of a full fossil record of viruses has led to various sources of information being used to gain an understanding of viral evolution. This section reviews some of these sources.

1. Viral Genome Sequences in Fossil Material

There are very few examples of evidence of viral genomes in fossils. Li *et al*. (1999) found provirus DNA of human T-cell lymphotropic virus type I (HTL V-I, *Retroviridae*) that closely matched with the modern HTLV-I in the bones of a mummy buried 1200 to 1500 years ago and preserved by the dry Atacama desert. The sequence of the tobamovirus, *tomato mosaic virus*, recovered from glacial ice cores approximately 140,000 years old in Greenland was nearly identical to contemporary isolates (Castello *et al*., 1999). However, there is some uncertainty as to whether the virus was contemporary with the ice.

There is the potential for obtaining older evidence of viruses in that nucleic acid sequences have been obtained from fossil plants and animals. For instance, a DNA fragment from the chloroplast gene *rbc*L in fossil *Magnolia latahensis* found in the Clarkia deposit dated as 17–20 mya (Miocene) (Golenberg *et al*., 1990), DNA was isolated from an amber-preserved bee (25–40 mya) (Cano *et al*., 1992), and DNA sequences were amplified by polymerase chain reaction from a fossil termite in Oligo-Miocene amber (DeSalle *et al*. 1992).

2. Virus-Induced Cellular Structures in Amber and Other Fossil Material

One aim of this and previous papers (Lovisolo and Rösler, 2001, 2003) is to make paleontologists using microscopy on fossils, mainly of plants and arthropods, aware of the possibility of detecting traces of virus infections.

Good preservation of virus-associated cellular structures would be given in amber, volcanic ashes, carbon deposits, gypsum crystals, and pits. Amberization gives the most complete type of fossilization of insects, enabling preservation not only of cells and tissues, but also of organelles such as mitochondria and ribosomes (Poinar, 1993).

Poinar and Hess (1982, 1985) investigated the ultrastructure of 40-million-year-old fossil fly tissues in Baltic amber, showing a good preservation of cell structures, including muscle fibrils, mitochondria cristae, and endoplasmic reticulum cisternae. Calcium carbonate permineralization gives good preservation of anatomical structures of vascular plants of Late Permian (250–270 mya) (Li *et al*., 1996). Tracheid and vessel elements were identifiable by light microscopy, whereas vessel perforation plates were recognizable by scanning electron microscopy.

Leaf galls have been reported on Paleocene and Eocene fossils (Wilf and Labendeira, 1999). Insects, mite, bacteria, and fungi cause the majority of plant galls; some viruses also cause them. Small phloematic galls, called enations, are caused by Fijiviruses in Gramineae, others bigger, present mainly on the roots, are caused by *Wound tumor virus (Phytoreovirus*).

There is direct fossil evidence of pathogens or symbionts of arthropods, such as bacteria, rickettsiae, cyanobacteria, protozoa, algae, and fungi (see Poinar, 1992, 1996; Poinar and Poinar, 1998, 1999; Poinar *et al*., 1993), but not yet for viruses infecting them. Fossil bacteria are rather difficult to find, but the study of bacterial symbionts of insects may help (Section III,C).

Although it is hard to detect isolated virions in fossil material, it could be easier to find traces of virus infection recognizable through structures related to infections that are larger than the virus particles.

Virus infection of plant and animal cells often causes virus-induced cellular structures (VICS), which are distinctive for some types of virus and recognized easily by electron microscopy and sometimes even by light microscopy (Lovisolo and Rösler, 2001, 2003). The main VICS are as follows.

1. Different types of inclusion bodies (protein structures staining abnormally, visible by light microscopy). For instance, among the viruses that infect angiosperms, typical "pinwheel" inclusions associated with potyvirus and several other types of inclusions could be recognized easily. Among the viruses that infect fungi, big lamellar inclusions could be found (see Lovisolo and Rösler, 2001).

2. Occlusion bodies (virions encapsulated in protein, sometimes large proteinaceous polyhedra). Examples include the large particles of *Entomopoxvirinae* species that are occluded in proteinaceous masses (Kurstak and Garzon, 1977) and the polyhedral and granular inclusion bodies of members of *Baculoviridae* (see Miller, 1996; Rohrmann, 1999; Winstanley and O'Reilly, 1999).

3. Membrane-bound enclaves of virions. Poinar and Poinar (1999) noted that entombed braconid wasps might possess polydnaviruses, which have membrane-bound particles.

4. Crystalline arrays of numerous virions. Larvae of Diptera, Lepidoptera, and Coleoptera infected with *Iridoviridae* produce closely packed paracrystalline arrays of virus particles (120 × 200 nm), mainly in the cytoplasm of fat bodies and of hemocytes (see Webby and Kalmakoff, 1999; Williams, 1996).

5. Viroplasms (amorphous cytoplasm inclusions associated to virus synthesis, without a surrounding membrane). Virus infections of tobacco plants are characterized frequently by the presence of large proteinaceous inclusion bodies, e.g., the X-bodies of *tobacco mosaic virus*, the inclusion bodies of caulimoviruses.

6. Protein tubular or lamellar structures. The neoplastic tissues of plants infected with some reoviruses are often full of virus particles, sometimes arranged in big crystals, viroplasms, and other virus-induced structures such as tubules (see Lovisolo and Rösler, 2001). Plant *Reoviridae* also replicate in their insect vectors and produce the majority of structures found in infected plants in several of their organs, for example, a microcrystal of a *Wound tumor virus* in muscle fibrils of a leafhopper (Shikata and Maramorosch, 1965). Lovisolo and Rösler (2003) compared electron micrographs of those muscle fibrils with the ones of a 40-million-year-old fossil fly embedded in amber.

3. Virus Particle Morphology

The majority of viruses comprise nucleic acid genomes encapsidated in a coat of a virus-encoded protein(s) together sometimes with lipids. The coat protects the genome on transmission between hosts, is often involved in the infection process, and can have other functions (see Section III,B). There are three basic shapes of virus particles: isometric, rod shaped, and complex; complex virus particles often have structural features related to those of isometric viruses (see Hull, 1976). In most cases, the arrangement of protein subunits in the viral capsid reflects an optimum energy state. For instance, most isometric viruses particles have icosahedral symmetry, with the subunits arranged in a quasiequivalent manner (Caspar and Klug, 1962). It is likely that solving the problem of achieving this optimum energy state occurred only once or very infrequently and that it represents an ancient feature of viruses.

Details of the coat protein structure and sequence can also provide evidence of viral evolution. The highly conserved β-barrel motif of the capsid protein of viruses with isometric particles indicates that many

viruses may have evolved from a single origin (Luo, 1999; Miles and Davies, 2000). However, one must not neglect the possibility of convergent evolution, as the β-barrel structure is the most efficient way of forming a protein subunit of an isometric particle. By extrapolation of the differences in coat protein sequences of the rod-shaped particles of seven tobamoviruses, Gibbs (1980) calculated that prototobamoviruses probably arose about 120–600 mya. Gibbs (1999) and Gibbs *et al*. (1999) observed that it is significant that the 19 genome sequences that they compared fall into lineages that mostly correlate with the family of the host from which they were isolated. They consider this significant in relation to the evolution and origin of tobamoviruses.

4. Genome Sequences

There has been a major surge in the sequencing of viral genomes since the late 1980s and sequences are available for representatives of most viral genera. Comparisons of viral genome sequences and organization are providing important information for understanding the evolution of viruses and possibly the origin of some virus groups. Comparisons among viruses in different phyla of hosts may also help in understanding the evolutionary origin of some host phyla. One difficulty in this approach to both animal and plant viruses is that the knowledge is not proportional to the distribution of the virus species in the different phyla. The knowledge is derived mainly from viruses of hosts important to humans and, as noted in Section I, is lacking for many significant phyla.

Despite these limitations, the great amount of information that is now accumulating is revealing possible evolutionary pathways for different genome types. These are too extensive to review here in detail and so we will exemplify them with a discussion of (+)-strand RNA viruses.

Virus-encoded replicases of (+)-strand RNA viruses comprise combinations of several enzymic activities, the RdRp, a helicase, and enzymes involved in "cap" (m^7G^5pppG) formation (methyl transferase, RNA 5′ triphosphatase and guanyltransferase). Sequence comparisons of RdRps of a large number of viruses show that they fall into three supergroups based on sequence motifs (see Koonin and Dolja, 1993). Similarly, viral helicases have been grouped into three superfamilies (Gorbalenya and Koonin, 1993). There is a divergence of opinion as to whether there is a monophyletic or polyphyletic origin of these supergroups and superfamilies (Gorbalenya, 1995; Zanotto *et al*., 1996).

Analysis of the replicases of a range of (+)-strand RNA viruses show that there is a wide variety of mixtures and arrangements of the sequences encoding these (and other) enzyme activities (Koonin and Dolja, 1993). It would appear that modern versions of the core "cassettes," comprising the replicase and, some cases, other genes such as coat protein, arose by molecular shuffling of the various components to give three major classes of viral RNA replicases. Koonin and Dolja (1993) proposed an ancestral form for each of these three classes of core "cassettes," suggested two alternative scenarios by which these could have arisen from a common ancestor, and went on to propose various routes by which the modern arrangements of the elements within the replicase could have arisen.

The aforementioned scenarios cover suggestions for the development of replication "cassettes." Tentative evolutionary pathways have been suggested for the accumulation of all the gene "cassettes" for the members of various virus families. This is exemplified by the model proposed by Dolja *et al*. (1994) for the complex RNA genomes of members of the *Closteroviridae*. It is suggested that the genomes of members of this family arose from a class III common replication "cassette" ancestor and that the current genome arose by various steps, including a split of the genome into two components to give crinivirus genome organization. It is suggested that the first two steps occurred early in evolution, giving a common ancestor for the whole tobamovirus cluster.

These examples illustrate the power of analysis of viral genome sequences. However, in the absence of direct fossil data, it must be remembered that the evidence is circumstantial.

5. Integrated Sequences

Viruses that integrate their sequences into the host genome may be regarded as an ultimate in coevolution. However, the situation is not as clear-cut as that. It is becoming increasingly apparent that pieces of any extrachromosomal DNA present in the nucleus of a eukaryote are likely to become integrated into the host chromosome. Viral DNA sequences are maintained through generations of host DNA replication and even through the evolution of the host that are of interest in discussing coevolution.

There are three situations in which viral sequences are found integrated into their host genome: viruses that require integration for their replication, viruses that replicate from both integrated and chromosomal forms, and integrated viral sequences that are not involved in, or capable of, replication.

a. Integration Essential for Replication The replication of retroviruses (*Retroviridae*, *Pseudoviridae*, and *Metaviridae*) involves reverse transcription of the virion RNA into DNA, integration of that DNA into the host genome, and transcription from that DNA to form progeny viral RNA. Various features of the viral genome mediate the integration, including the reverse transcription phase, giving linear DNA with long terminal repeats and the encoding of an integrase. This form of replication and the genomic features are also found in a variety of retroelements that differ from retroviruses only in that they do not form particles and are not transmitted horizontally. Thus, in discussion of viral coevolution, we have to also consider these retroelements.

Another group of reverse-transcribing viruses, the pararetroviruses (*Hepadnaviridae* and *Caulimoviridae*), replicate without integration. The DNA genomes of pararetroviruses form extrachromosomal "minichromosomes," which are the templates for transcription, and the resulting RNA is transcribed into DNA. These genomes do not contain the gene for an integrase, and the reverse transcription phase of replication results in a circular double-stranded DNA (without long terminal repeats). The foamy viruses (genus *Spumavirus*) appear to have properties of both retroviruses and pararetroviruses and thus lie between the two groups (Lecellier and Saïb, 2000).

One other virus family, the *Polydnaviridae*, replicates from integrated DNA that is located at multiple sites in the genomes of parasitic wasps. Viral genome replication involves the excision of viral DNA segments by site-specific recombination events.

b. Viruses That Replicate from Both Integrated and Extrachromosomal Forms Several families of viruses replicate their DNA genomes from both integrated and extrachromosomal forms. In temperate phage, such as members of the *Myoviridae*, *Lipothrixviridae*, and *Inoviridae*, there can be both lytic, extrachromosomal replication and integration of the viral genome (prophage) that can be activated to give lytic infection.

As well as being able to replicate extrachromosomally, the DNA of members of the vertebrate-infecting *Polyomaviridae* integrate to give a latent infection. This integrated DNA can be activated later and replicate extrachromosomally, giving the full symptoms of the disease.

For many years it was thought that plant viruses did not integrate into the host genome. However, three members of the *Caulimoviridae* have been recognized to have integrated forms that can be activated by stresses to give episomal infections (reviewed by Harper *et al*., 2002).

Most of the detailed studies have been undertaken on *Banana streak virus* (BSV). It appears that the integrated form of the BSV genome in certain banana cultivars (AAB genome) is complex, being composed of multiple copies, arranged in tandem, of sequence that could yield the viral genome. Each copy comprises the BSV genome interrupted by an insert composed of a highly rearranged BSV sequence. It is suggested that the episomal replicating form of the virus can be derived from the integrant by two recombination events (Harper *et al*., 1999; Ndowora *et al*., 1999).

c. Integrated Viral Sequences That Do Not Replicate Infection with many DNA viruses gives rise to fragments of the viral DNA integrated in the host genome. Eukaryotic genomes contain several families of sequences with homology to retroviruses, termed LTR retrotransposons. These endogenous retrovirus-like elements (ERV-L) are widespread in placental mammals, suggesting that they were already present at least 70 mya (Bénit *et al*., 1999). Studies on these ERV-L elements, especially the human equivalents (HERVs), show that they contain at least two, and sometime three, of the canonical retroviral genes (*gag*, *pol*, and sometimes *env*) (Löwer *et al*., 1996). In most cases, the coding capacity of these elements is disrupted by mutations or recombinations and so they would appear not to be active as viruses. They are considered intermediate between classical intracellular retrotransposons and infectious retroviruses, but there is no evidence as to which is the progenitor of the other. Fragments of the sequence of the pararetrovirus *Hepatitis B virus* are found integrated in the host genome.

In plants, geminivirus-related sequences have been found integrated into various *Nicotiana* species (Ashby *et al*., 1997; Bejarano *et al*., 1996). No exogenous retroviruses have been reported from plants, but analysis of the *Arabidopsis* genome suggested that a retrotransposon, Tat 1, contained a gene for an *env*-like protein, a characteristic of retroviruses (Wright and Voytas, 1998). As noted previously, several members of the pararetrovirus *Caulimoviridae* integrate in an activatable form into the host genome. There is now increasing evidence for inactive ("dead") pararetroviral sequences in plants. Host genome sequencing has revealed endogenous pararetroviral-like (EPRV-L) sequences in tobacco (tobacco pararetrovirus, TPV) and rice (Jakowisch *et al*., 1999; Mao *et al*., 2000); TPV is related to *Tobacco vein clearing virus*, an activatable integrating virus, and that in rice to *Rice tungro bacilliform virus* (RTBV), not known to integrate in an activatable form. Geering *et al*. (2001) reported nonactivatable "dead" integrants

of BSV from a range of banana cultivars. Using primers based on the TPV sequence designed to reveal pararetrovirus-like sequences, Hansen (2003) detected EPRV-L sequences in plant species from a diverse range of phyla, including liverworts, ferns, gymnosperms, and monocotyledonous and dicotyledenous angiosperms.

d. Discussion Modern techniques have great potential for providing data on the coevolution of viruses and their hosts. Although currently there is a lack of conventional fossil evidence on virus origins and evolution, there is a considerable amount of circumstantial data. The only problem with interpreting these data is that they are not arranged "stratigraphically" and thus it is not possible to gain evidence on which feature arose before which feature.

There are potential sources of direct fossil evidence of viruses, some of which were discussed earlier. Various materials have been found preserved in ice. Analysis of living bacteria in glacial ice cores up to 20,000 years old (Christner *et al*., 2000) may yield information on phage; similarly, various creatures and plants have been found in permafrost that was frozen up to 40,000 years ago. Studies of life forms in Lake Vostok, which was capped by Antarctic ice more than 420,000 years ago, may show evidence of virus infections. Willerslev *et al*. (2003) demonstrated that plant and animal DNA may be preserved, at least in permafrost and dry cave soils, for up to 400,000 years. However, most of these approaches are relatively recent in evolutionary terms, although they should give information on viruses before the impact of humans. For early events in virus coevolution, we have to refer to molecular "fossils," especially the relationships that integrated sequences have with each other, coupled with an understanding of the evolution of their hosts, which can give some important leads.

Homologous sequences in nonstructural proteins of positive single-stranded RNA plant and animal viruses and similarities in the genome expression strategies led to the suggestion that some plant viruses could have common ancestors with some animal viruses (see Hull, 2001).

Most of the integrated sequences are reverse-transcribing elements and, as reverse transcriptase is likely to have evolved early to mediate the movement from the "RNA world" to the "DNA world," it is likely that some of these integrated elements represent old events. As well as the LTR retrotransposons described earlier, non-LTR retrotransposable elements are found in eukaryotic genomes. Phylogenetic analyses of these non-LTR retrotransposable elements suggest that they arose before LTR retrotransposons and are possibly as old as

eukaryotes dating back to the Precambrian era (Malik and Eickbush, 2001; Malik *et al.*, 1999). An analysis of the RNase H domain suggested that LTR retrotransposons arose from the non-LTR version and these then gave rise to retroviruses (Malik and Eickbush, 2001). However, this scenario is complicated by the enzyme that repairs chromosome termini, telomerase, which resembles RT in several respects. It is thought that this enzyme might have been involved in the development of LTR retrotransposons (and retroviruses) from non-LTR-retrotransposable elements (Eickbush, 1997).

It is not clear how the evolutionary pathways of retroviruses and pararetroviruses relate, but there are several points that should be kept in mind. In general, retroviruses are found in animals and pararetroviruses in plants. Although hepadnaviruses, which infect animals, are regarded as pararetroviruses because they replicate extrachromosomally, they differ from retroviruses and plant pararetroviruses in important details of their replication. The division of kingdoms in hosts of retroviruses and pararetroviruses might suggest that they evolved separately after plants and animals diverged. This could be supported by the tRNA primer for (−)-strand DNA synthesis, which is conserved as $tRNA_{met\ I}$ for plant pararetroviruses and retroelements and a variety of tRNAs for animal retroviruses (Hull and Covey, 1996). However, there are two observations that make this scenario less clear. First, there is the occurrence, noted earlier, in the *Arabidopsis* genome of a retrotransposon, Tat1, containing a gene for an *env*-like protein, a characteristic of retroviruses (Wright and Voytas, 1998). Second, some retrotransposons in plants have a different tRNA primer (see Ohtsubo *et al.*, 1999).

It is generally accepted that active integrating retroelements, be they viral or nonviral, play a part in evolution of the host (Bennetzen, 2000; Schmidt, 1999). However, the question arises as to why so many integrated elements are retained when they appear to have lost the ability to replicate and transpose or even to express proteins. One possible answer comes with the increasing understanding of gene silencing and suggests that they play a role in defense systems against "foreign" nucleic acids (Hull *et al.*, 2000; Matzke *et al.*, 2000; Mette *et al.*, 2002). This would be an obvious role for "dead" integrated viral sequences.

IV. Virus–Host–Vector Coevolution

The previous two sections considered the evolution of hosts and viruses. This section discusses how their evolutions interacted.

As noted earlier, some Insecta and Acarina species are major vectors of viruses of both plants and animals. They are much diversified in the ways they transmit viruses with the most complex relationships being between insects and plant viruses. It is generally recognized that insects have played an important role in the evolution of angiosperms, mainly due to their involvement in pollination and with significant insect herbivory begining during the Late Carboniferous to Early Permian (Labandeira, 1999). These relationships have certainly favored the evolution of plant viruses.

Thus, arthropods are pivotal in the discussion of coevolution of the virus–host vector. In this discussion, several factors have to be considered.

1. Virus classification involves a range of virus properties, including shape and structure of the virus particle, type and size of nucleic acid, and genome organization, as well as the biological properties of host range and transmission.
2. When viewed from the point of view of virus classification, there are several categories of virus–vector interactions: (a) virus families in which species replicate in arthropods and some in plants and others in animals; (b) virus families in which species replicate in arthropods and in either plants or animals; (c) virus families that replicate in plants or animals and are transmitted by arthropods in which they do not replicate; and (d) virus families and genera that replicate only in arthropods.
3. The mechanisms by which viruses gain entry to and spread from cell to cell in animals and plants. Entry and cell-to-cell spread of animal viruses (both of vertebrates and invertebrates) involve specific cell surface receptors that are recognized by a surface protein on the virus particle and/or fusion of a viral membrane with the cell plasma membrane. Entry of a virus into a plant involves mechanical damage of the surface layers of the plant (the cuticle and cell wall) with introduction directly into the cell. Cell-to-cell movement is via intercellular cytoplasmic connections, termed plasmodesmata, which are often modified by one or more viral gene product.

Taking these factors into consideration in the following four subsections, we discuss the categories defined under the second factor.

A. Replication in Arthropods, Plants, or Animals

Three virus families, *Bunyaviridae*, *Rhabdoviridae*, and *Reoviridae*, contain species that replicate in arthropods and also in either plants or animals. Particles of members of the first two families are enveloped

with glycoprotein spikes that recognize animal cell surface receptors protruding through the membrane; the nonenveloped particles of *Reoviridae* have either surface projections (*Orthoreovirus, Cypovirus, Aquareovirus, Fijivirus*, and *Oryzavirus*) or surface proteins (*Orbivirus*, *Rotavirus*, *Coltivirus*, and *Phytoreovirus*) that interact with animal cell surface receptors. These cell surface receptors appear to have no relevance to infection of plants.

Bunyaviridae infect mainly vertebrates. Members of the genera *Bunyavirus, Nairovirus*, and *Phlebovirus* replicate in vertebrates and arthropods (Diptera, Hemiptera, and Ixodidae); no vectors are known for the Hantaviruses. *Bunyavirus*, *Nairovirus*, and *Hantavirus* genera have genomes comprising three segments of (−)-strand RNA and encode three open reading frames; the genomes of phleboviruses comprise two (-)-sense RNAs and one ambisense-RNA, thus having one more open reading frame than the other three genera (Fig. 2A).

The family *Bunyaviridae* has one genus infecting plants (*Tospovirus*), the members of which replicate in, and are vectored by, *Thripidae*. As with vertebrate-infecting bunyaviruses, tospovirus particles are enveloped with glycoprotein surface projections. Tospovirus genomes comprise one segment of (−)-strand RNA and two segments of ambisense RNA (Fig. 2B), thus containing one more open reading frame than the related phleboviruses.

dsRNA *Reoviridae* have five genera of viruses infecting vertebrates (*Orthoreovirus*, *Orbivirus*, *Rotavirus*, *Coltivirus*, and *Aquareovirus*) and three infecting plants (*Fijivirus*, *Phytoreovirus*, and *Oryzavirus*). All three of the plant-infecting genera replicate in their insect vectors (Delphacidae and Cicadellidae), but of the animal-infecting reoviruses, only *Orbivirus* and *Coltivirus* genera have members that replicate in diptera and in ticks (Argasidae and Ixodidae), with little or no evident effect.

Orthoreoviruses and rotaviruses are found only in warm-blooded vertebrates and aquareoviruses in poikilothermic vertebrates and in invertebrates such as crustacea; the genus *Cypovirus* is limited to arthropods.

Even in reovirus genera that contain species that infect both arthropods and vertebrates or plants there are species that are found only in arthropods. Thus, orphan orbiviruses of arthropods are known. A few Fijiviruses are orphans in Delphacidae, such those found in *Peregrinus maidis* (Delphacidae, Hemiptera) by Falk *et al*. (1988) and Herold and Munz (1967). Other reoviruses that infect only arthropods are the leafhopper A virus and the *Niloparvata lugens reovirus*.

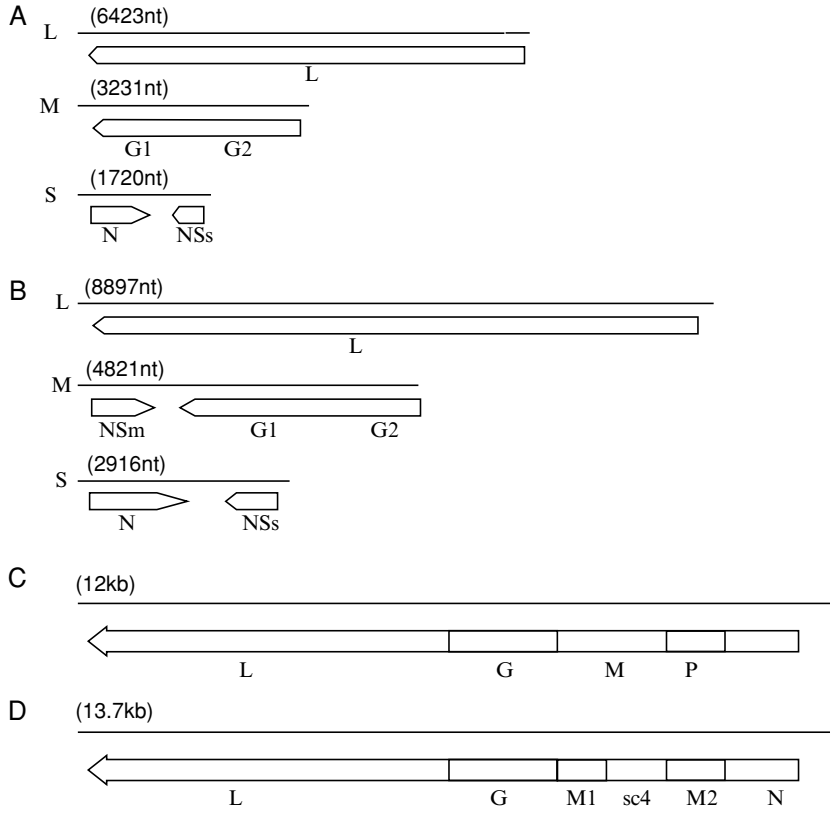

FIG 2. Genome organizations of A and B Bunyaviruses, (A) *Uukuniemi virus* (phlebovirus) and B *Tomato spotted wilt virus*, (C and D) Rhabdoviruses, (C) *Vesicular stomatitis virus*, (D) *Sonchus yellow net virus*. For each, single lines represent the genomic RNAs (with the designations and sizes indicated) and arrowed boxes represent translation products; those for rhabdoviruses are divided to show the final products. Below the boxes are designations for the final products: G, glycoprotein; L, large protein; M, matrix protein; N, nucleocapsid protein; Ns, nonstructural protein.

The genomes of *Reoviridae* comprise 10–12 segments of dsRNA, the actual number being characteristic of the genus. At least one of the RNA segments is dicistronic.

Rhabdoviridae comprise six genera, two of which (*Vesiculovirus* and *Ephemerovirus*) infect arthropods and vertebrates, two (*Cytorhabdovirus* and *Nucleorhabdovirus*) infecting arthropods and plants, one (*Lyssavirus*) infecting only warm-blooded vertebrates, and one (*Novirhabdovirus*) infecting fish. Numerous animal and plant rhabdoviruses

are unassigned to any genus and some appear to essentially be nucleoprotein "cores" (Francki *et al*., 1985). A few of the unassigned plant rhabdo-like viruses are transmitted by *Brevipalpus* mites. These include Orchid fleck virus (OFV) and Citrus leprosis virus, which have nonenveloped particles, being just nucleoprotein "cores" (Lovisolo, 2001). The genome of OFV comprises two single-stranded RNA molecules, and one of the encoded proteins has similarities to the polymerase of some plant and animal rhabdoviruses (Kondo *et al*., 1998).

The genomes of rhabdoviruses are (−)-strand RNA, which is transcribed into generally monocistronic mRNAs. The genomes of vertebrate-infecting rhabdoviruses are expressed as five primary proteins (Fig. 2C) and those of plant-infecting rhabdoviruses as six proteins (Fig. 2D).

Members of animal-infecting vesiculoviruses and ephemeroviruses may be transmitted by hematophagous arthropods, such as phlebotomine sandflies and mosquitoes, and some vesiculoviruses may be vectored by fish ectoparasites. Plant *Rhabdoviridae* have vectors belonging mainly to leafhoppers (Cicadellidae) or planthoppers (Delphacidae) and aphids. Few species are transmitted by tingid bugs (Tingidae) and mites (Brevipalpidae).

Viruses of the three families (*Bunyaviridae*, *Rhabdoviridae*, and *Reoviridae*) described earlier that replicate in both arthropods and plants and/or animals represent a good example of coevolution of virus–host–vector. They have been considered to be viruses of arthropods that have become adapted to their plant or animal host.

In analyzing them, three features emerge.

1. Within each family there is a variation on a theme. The theme is criteria such as particle morphology and composition and genome type and basic organization that delimit the family. The variation is that within the family there are genera and/or species that replicate both in arthropods and animals or plants and others that are limited to either arthropods or animals or plants.

2. As noted earlier, the basic mechanisms of infection of and spread within arthropods and vertebrates are very similar, involving cell surface recognition proteins on the surface of the virus particle. Infection of plants requires mechanical damage to insert the virus particles through the cuticle and cell wall into the cell; this can be affected by sap-sucking insects. However, cell-to-cell spread is mediated by a virus gene product(s) that modifies plasmodesmata, gene products not required in animals. These have been identified in at least two of the plant-infecting members of the families described earlier. In

tospoviruses, NSm, encoded by the ambisense M RNA segment (Fig. 2B), has been shown to be involved in cell-to-cell spread (Storms, 1998). It is of interest to note that analogous RNA of the related animal-infecting phleboviruses is not ambisense but (−)-strand (Fig. 2A). Plant-infecting rhabdoviruses also express a protein (sc4) not found in animal-infecting rhabdoviruses, which is thought to be involved in cell-to-cell spread (Fig. 2D) (Jackson *et al*., 1999).

Realization of a further adaptation to plants is emerging. Plants have a defense mechanism against "foreign" nucleic acids, termed RNA silencing, that involves the degradation of viral and other RNAs. Viruses have overcome this by encoding a protein(s) that suppresses this defense (for a review, see Baulcombe, 2002). However, this defense system, and the suppression of it, may not be restricted to plant viruses, as an example of the phenomenon has been reported for the animal virus, *Flock house virus* (FHV) (Li *et al*., 2002).

3. The replication of viruses involves intracellular membranes, and differences exist between animals and plants in the subcellular organization of these membranes. There is little information on this, but it might be reflected in the case of *Reoviridae*, where it is suggested that the genes for replication in the plants are different from those required for replication in insects (Nuss and Dall, 1990).

B. Replication in Arthropods and either Plants or Animals

This second category overlaps the first but differs in identifying virus families that are limited to arthropods and either vertebrates or plants. As discussed later, it is possible that the evolutionary considerations are different from those involved in the virus families described in Section IV,A. There are two overlapping subsets of this category: families that contain genera that replicate in both animals and their arthropod vectors and families that comprise some genera that infect animals or plants and other that infect arthropods.

The family *Flaviviridae* is composed of three genera: *Flavivirus*, members that replicate in both vertebrates and arthropod vectors (mosquitoes and ticks), and *Pestivirus* and *Hepacivirus*, which only replicate in vertebrates. The family *Orthomyxoviridae* contains one genus (*Thogovirus*), which replicates in both vertebrates and the arthropod vector, and three genera (*Influenzavirus A*, *Influenzavirus B*, and *Influenzavirus C*) that infect only vertebrates. There are no members of either of these families that infect plants.

Families that contain genera that infect only vertebrates and others that infect only arthropods include *Birnaviridae, Iridoviridae, Nodaviridae, Parvoviridae, Poxviridae*, and *Togaviridae*.

There is uncertainty as to whether the plant-infecting *Maize rayado fino virus* (genus *Marafivirus*) and *tomato yellow leafcurl virus*–Israel (genus *Begomovirus*, family *Geminiviridae*) replicate in their respective vectors, the leafhopper (*Dalbulus maidis*) and the whitefly (*Bemisia tabaci*) (Ammar and Nault, 2002; Brown and Czosnek, 2002).

The description in this section of the interactions between viruses and hosts from various kingdoms raises a major point. As noted previously, the classification of viruses is based on various criteria, especially features of the particle and genome. Thus, it reveals genera of a family that are limited either to arthropods plus vertebrates or to arthropods or vertebrates. This may reflect the adaptation of a prototype of the family to different kingdoms.

Metaviridae and *Pseudoviridae* represent a very different situation in that the viral sequences are integrated into the host genome and the viruses are transmitted vertically, there being no evidence for horizontal transmission. Species of one of the genera (*Metavirus*) within the *Metaviridae* are found in either higher plants or yeast and those in the other genus (*Errantivirus*) in insects and nematodes. In *Pseudoviridae*, one genus (*Pseudovirus*) has species in higher plants or yeast and another genus (*Hemivirus*) in insects or yeast. Genome sequencing shows that mutated sequences of these viruses are also incapable of replication. These "dead" viral sequences are important in the consideration of coevolution (see Section III, D,5).

C. Replication in Plants or Animals but Not in Arthropod Vectors

Arthropod transmission of viruses that replicate in vertebrates but not in the vector is very rare. *Lumpy skin disease virus* (family *Poxviridae*; genus *Capripox*) is transmitted in a "mechanical" manner by the mosquito *Aedes aegypti* (Chihota *et al*., 2001). However, evidence shows that the mode of transmission is more complex than a mere "dirty pin," but there is no information available on the specificity of any interaction.

A few very infective and stable plant viruses, such as some tobamo, potex, and tymoviruses, are also transmitted in an essentially mechanical means by insects with a chewing feeding behavior such as Orthoptera and Dermaptera. This interaction could have had some role in the past for the natural transmission and evolution of some viruses. Interactions that are more specific are found with some viruses transmitted

by Coleoptera, Orthoptera, and Dermaptera. In these, there appears to be some specificity in using a chewing–regurgitating feeding mechanism for transmission but there is little specificity for the insect order.

Many plant viruses have very specific interactions with the invertebrate vectors in which they do not replicate. These interactions fall into two basic types: externally borne (also termed nonpersistent, semipersistent, or stylet-borne) in which the virus associates specifically with the mouthparts or exoskeleton and internally borne circulative (also termed persistent or circulative) in which the virus passes through the gut wall, into the salivary glands, usually via the hemocoel, from where it is introduced into the healthy plant. The basic concepts of these interactions were developed by Watson and Roberts (1939) (introducing the terms nonpersistent and persistent) for the aphid transmission of plant viruses, but now these terms can also be applied to transmission by other arthropods, nematodes, and fungi. These interactions are discussed in detail by Hull (2001) and reviewed in chapters in Plumb (2002).

Two forms of virus–vector molecular interactions are found in externally borne transmission. For some viruses, such as *Alfalfa mosaic virus* and *Cucumber mosaic virus*, the interaction is directly between the surface sequence or structural features on the virus coat protein and specific sites on the aphid vector stylets (Bowman *et al*., 2002; Liu *et al*., 2002). The other form is an indirect interaction involving a virus-coded protein termed a helper component, helper factor, or transmission component. In this interaction, the helper component binds to both the virus coat protein and the specific sites in the aphid stylets. Molecular interactions have been described for three virus genera that are transmitted in the externally borne manner (Blanc *et al*., 2001; Raccah *et al*., 2001). The potyvirus helper protein, HCPro, and the caulimovirus helper protein, P2, are unique, bearing little resemblance to other virus-encoded proteins. The helper component of closteroviruses is suggested to be a modified coat protein expressed from a separate open reading frame to the major coat protein and which forms a "tail" on virus particles (Agranovsky *et al*., 1995; Febres *et al*., 1996); it is interesting to note that this modified coat protein is also involved in cell-to-cell movement of the virus (Dolja, 2003).

Internally borne plant viruses comprise those that replicate in their vector and those that do not replicate; the replicating viruses were discussed in Section IVA. Nonreplicating viruses have some features in common with those that replicate in their vector to enable the virus to pass through the arthropod gut and enter the salivary glands. The current understanding of the interaction involved in the passage of

luteoviruses across the gut wall cells and into the salivary glands was described by Gildow (1999), Herrbach (1999), and Reavy and Mayo (2002). There was a suggestion that a read through from the coat protein open reading frame (P5) was involved in cell surface recognition (Guilley *et al*., 1994). However, evidence, including the possible interaction of P5 with GroEL (described in Section III,C) in protecting virus particles, suggests that the role of P5 is complex (discussed by Reavy and Mayo, 2002; Taliansky *et al*., 2003).

D. Replication Only in Arthropods

Members of four virus families (*Ascoviridae, Baculoviridae, Polydnaviridae*, and *Tetraviridae*) and one unplaced genus (CrPV-like) are restricted to arthropods.

Ascoviridae (dsDNA) and *Tetraviridae* (ssRNA) infect Lepidoptera and are mostly transmitted mechanically, in the case of ascoviruses by ectoparasitic wasps. An ascovirus that infects *Acrolepiopsis assectella* (family Yponomeutidae) is transmitted by the ichneumonid wasp *Diadromus pulchellus*, with the viral genome being carried in the wasp nuclei. Polydnaviruses are found in ectoparasitic hymenopterous insects (Ichneumonidae and Baconidae).

Members of *Baculoviridae* and CrPV-like viruses have hosts in a number of insect families, and Baculoviruses have also been reported from the crustacean order Decapoda (shrimp).

Numerous other ssRNA viruses, mainly arthropod picorna-like (Moore *et al*., 1985; Scotti and Christian, 1999), have been isolated from insects (Orthoptera, Hymenoptera, Lepidoptera, Hemiptera, Diptera, Isoptera) and acari (*Panonychus* spp., Tetranychidae and *Varroa jacobsoni*, Varroidae). The amyelosis chronic stunt virus, which multiplies in insects, is an unassigned species of *Caliciviridae,* a family of vertebrate viruses. In addition to being hosts of picorna-like viruses, Hemiptera *Myzus persicae*, *Rhopalosiphum maidis*, *R. padi*, and *Bemisia tabaci*, are well known vectors of plant viruses.

E. Discussion

The coevolution of virus–host–vector has several facets.

• Adaptation of the virus to hosts from different kingdoms, usually arthropods and vertebrates or arthropods and plants.

• The usual perception is that the arthropod is the vector of the virus between vertebrate or plant hosts, but it can also be considered that the vertebrate or plant host "vectors" the virus between arthropod hosts.

• The virus has to overcome the host defenses, but as pointed out in Section III,B, it is essential for the virus to damage the host, be it arthropod, vertebrate, or plant, as little as possible. For instance, vesiculoviruses infect mammals, insects, and ticks. Shope and Tesh (1987) reported that infection of vertebrate cells with members of the *Vesicular stomatis virus* sero-group results in rapid and massive cell destruction, whereas their growth in insect cell cultures is characterized by the lack of cytopathic effect and by persistent infection. This is a further indication that insects are probably older hosts of vesiculoviruses than vertebrates. The *Vesicular stomatitis virus* multiplies efficiently when inoculated in *Peregrinus maidis* (Lastra and Esparza, 1976), an important vector of plant viruses.

• The basic replication cassette of the virus has to be linked to cassettes of genes and nucleic acid sequences that adapt it to the host. Many of these host-adapting cassettes are likely to be similar for viruses that infect arthropods and vertebrates, but those that infect plants require different genes for transmission, initial infection, and cell-to-cell spread.

Thus, from the coevolution perspective, the four categories of virus–vector–host interactions described earlier can be placed into three systems: coevolution of virus–host–vector, coevolution of virus and vector, and coevolution of virus and host.

As noted in Section IV,A, viruses of *Bunyaviridae, Rhabdoviridae*, and *Reoviridae* that replicate in both arthropods and plants and/or animals represent a good example of coevolution of virus–host–vector. However, as shown by plant rhabdovirus "cores," there appear to be examples of adaptation to one of the hosts. It could be possible that this is associated with the vegetative propagation of some plant species, which makes the need for horizontal transmission between hosts less necessary.

Flaviviruses that replicate in vertebrates and their arthropod vectors also fit into this system, although there are no examples of species that replicate in plants. In each case, the viruses have cassettes of specific genes that adapt them to their host.

Mutualistic virus–fungus–plant coevolution has evolved inside phytopathogenic fungi, as shown by the presence of virus-like dsRNA (Buck and Brasier, 2002; Tavantzis and Lakshaman, 1995). Cole *et al*.

(2000) detected RNA-dependent RNA polymerase activity in mitochondria from an isolate of *Ophiostoma novi-ulmi* infected with the naked RNA virus *Ophiostoma novi-ulmi mitovirus-6*. The involvement of mitochondria in virus replication is probably important in coevolution. Gibbs *et al*. (2000) suggested that these dsRNAs evolved from a defective ssRNA virus.

The coevolution of viruses that replicate in the vertebrate or plant host but not in the arthropod (Section IV,C) probably follows a different route to that of virus–vector–host as shown in bunyaviruses, rhabdoviruses, reoviruses, and flaviviruses. In this system, the plant or animal does not act as a "vector" for the arthropod virus. The viruses of this system have acquired one or more genes that adapt them to this mode of interaction.

Further factors appear to control the coevolution of viruses and hosts (Sections IV,B and D). If one takes "natural" infections, it would appear that viruses are capable of replication in only hosts of only one kingdom. However, experimental infections have shown that this is not necessarily true. The Nodavirus FHV (+ strand RNA) (formerly *Black beetle virus*) naturally infects insects but replicates in vertebrate, plant, and yeast cells under experimental conditions, although it requires special techniques to initiate the infection. It replicates in several plant species (Selling *et al*., 1990) and spreads systemically in *N. benthamiana* if complemented by the cell-to-cell movement proteins of *Tobacco mosaic virus* or *Red clover necrotic mottle virus* (Dasgupta *et al*., 2001). This indicates that, at least for FHV, the ability to replicate in hosts from other kingdoms is not a limit to its natural host range. It is likely that the limit is that this virus has not infected an arthropod that feeds on either plants or vertebrates. It is of interest to note that a related virus, *Nodamura virus*, replicates in mosquitoes and infects vertebrates. Thus, one important aspect of coevolution is the ability, or even the chance, of the virus entering the interaction between a haemotophygous or phytophygous insect and its vertebrate or plant source of food.

Do any of these three systems of coevolution give any pointers to the routes of coevolution? It would seem to be most likely that the virus–host–vector system originated from prototypes of the *Bunyaviridae, Rhabdoviridae*, and *Reoviridae* that infected early arthropods and became adapted to either vertebrates or plants. Generally, *Bunyaviridae* are cytolytic for vertebrates, but cause little or no cytopathogenicity in the arthropod hosts. Tospoviruses could have evolved as plant viruses within the *Bunyaviridae* through insects or acari (Roggero *et al*., 1995). Thrips attacking humans are known (Bailey, 1936) and

one species was identified (Arnaud, 1970) as *Frankliniella occidentalis*, an important vector of Tospoviruses.

The virus–host interactions of viruses that are propagative in arthropods probably played a role in the evolution of viruses not only of arthropods but also of vertebrates and plants (Nuttal *et al*., 1991). There appear to be two routes for interkingdom horizontal transfer of such viruses, from the insect feeding on the plant or animal and from the animal feeding on the insect. The *Iridoviridae* have only been isolated from poikilothermic animals (amphibians, fishes, and insects), and it has been suggested that insect viruses evolved first and that they later adapted to life in vertebrates that fed on the insects (Webby and Kalmakoff, 1999).

Jolivet (1998) noted that it seems that at the beginning all of those hematophagous bloodsucking insects were phytophagous or nectariphagous, as males mosquitoes still are. In addition, Calisher (1999) pointed out that mosquitoes belonging to some of the species that transmit alphaviruses typically feed on plants as well as on vertebrates.

Some viruses that replicate in arthropods had probably evolved as agents of diseases in vertebrates and plants, and some could have moved from vertebrates to plants and vice versa through arthropods. No rhabdovirus is known to be transmitted vertically in vertebrates or plants. This may be an indication that they originated in arthropods.

The origin of the virus–vector system would seem likely to be viruses of plants that have acquired properties that have facilitated specific interactions with insects. Some of the nonpropagative closteroviruses have vectors (treehoppers and mealybugs) that originated quite recently. Pseudococcidae originated in the Priabonian age and Membracidae in the Rupelian age, Tertiary; Miridae (Heteroptera) originated in the Oxfordian age of the Jurassic (146 to 154 mya) (Ross and Jarzembowski, 1993). However, the question arises as to how these viruses moved around before they acquired their insect transmissibility.

Disparities in the distribution of type of viral genomes among host kingdoms (Table I) might suggest that there might be kingdom constraints on the replication of different genome types. These might include the replication conditions and/or host factors that the virus might use.

On host conditions, it has been pointed out that viruses of plants and of poikilothermic animals have optimal replication rates mainly at temperatures lower than 30 °C, whereas homoiothermic animal viruses have replication optimum higher than 30 °C (see Lovisolo and Rösler, 2001). For instance, arthropod-infecting members of the

subfamily *Entomopoxvirinae* have an optimal growth temperature at 26–28 °C as compared to about 37 °C for poxviruses of the vertebrate (Moyer, 1999). Similarly, the replication of cypoviruses that infect Lepidoptera, Diptera, Hymenoptera, Coleoptera, Neuroptera, and a cladoceran crustacean is inhibited above 35 °C (Belloncik, 1999).

As to host factors, the situation with FHV, which can replicate in insect, vertebrate, plant, and fungal cells, shows that there appears to be no limitations for RNA-dependent RNA polymerase. Whether there are host factor limits to DNA-dependent DNA polymerase or reverse transcriptases is not known. Table I shows that once a genome type enters a kingdom, it can diversify in that kingdom.

V. Coevolution of Viruses and Hosts since Human Appearance

It is important to distinguish between the evolution of viruses and the evolution of virus diseases as discussed by Hull (2001), Lovisolo (1989–1990), and Nathanson *et al*. (1995). The evolution of viruses started very early in the development of life forms, whereas the evolution of virus diseases of both plant and animals is relatively recent, being mainly connected with human activity.

This section considers some of the impact that human activity has had on the coevolution of viruses, hosts, and vectors.

A. Evolution of Humans and Development of Agriculture

There is much controversy on human evolution, but it is now generally accepted that the split between hominids and living apes occurred about 5–10 million years ago. The hominids then evolved through several *Homo* spp. to become modern man, *Homo sapiens sapiens*, about 120,000 years ago. Until about 10,000–15,000 years ago, modern man was a hunter-gatherer and, as far as virus evolution is concerned, would have had no more impact on plants and animals than any other predator or herbivore. The development of agriculture about 10,000–15,000 years ago has had an increasing impact on the coevolution of viruses with their vectors and hosts. The development of agriculture was the intentional planting of crops, and a key step was the domestication of wild plant species and wild animals. It is now thought that domestication occurred at about the same time (around 10,000 years ago) at several places around the world. The main centers of domestication for different crops and animals were China, south and southeast Asia (India, Malaysia, and Indonesia), Ethiopia, the Middle East, the

Mediterranean region, and Central and South America. The major spread and movements of modern man, starting about 8000 years ago, led to the distribution of these crops from their centers of domestication. Improved travel and communication over the last 500 to 1000 years led to crops being grown wherever there are favorable conditions worldwide.

Advances in agriculture are considered one of the factors leading to the development of "civilization" and the divergence between urban and rural societies, which, in turn, have intensified agricultural practices. Thus, humans have had a major impact on the potential interactions of viruses, their vectors, and their hosts.

B. Examples of Impact of Human Behavior on Vertebrate Viruses

The main influences that humans have had on the interactions of viruses with vertebrates are derived from the domestication of mammals and birds together with the subsequent agronomic practices and human behavior associated with "civilization." These, and especially the latter, are extremely complex issues (see Osterhaus, 2001; Weiss, 2001) and are beyond detailed discussion in this review. However, two examples illustrate some of the factors involved, both of which show the anthropocentric nature of the issues.

Influenza is termed an "emerging" virus even though it was clearly described in the late 1100s (reviewed by Webby and Webster, 2001). This is because *Influenza A virus* undergoes genetic drift and shift. The former, caused by mutations in significant genes, can be said to be driven by the concentration of its hosts into centers such as cities where transmission is easy and there are many hosts. The latter is caused by reassortment of the genome segments of different strains of the virus. There is increasing evidence of gene flow through reassortment from avian to human hosts of the virus. Analysis of several influenza epidemics and pandemics indicates that the close proximity of avian hosts (e.g., chickens, ducks, and quail), pigs, and humans has led to new strains of the virus virulent in humans (see Hay *et al*., 2001; Webby and Webster, 2001). In this scenario, pigs play a pivotal role in that they are susceptible to both avian and human strains of the virus and it is likely that it is in this host that recombination takes place.

It is generally accepted that the acquired immune deficiency disease (AIDS), which over the last 20 or more years has resulted in tens of millions of deaths, is caused by *Human immunodeficiency virus* (HIV). There is much speculation as to the origin of HIV (reviewed in papers edited by Weiss and Wain-Hobson, 2001). The current evidence

is that HIV-1 is closely related to the simian virus from west African chimpanzees, SIVcpz, and that HIV-2 is related to a virus from sooty mangabeys (SIVsm) (reviewed in Sharp *et al*., 2001). Simian viruses are benign in their natural hosts.

There are two areas of human influence in the AIDS epidemic. The first is as to how these viruses crossed the species barrier. Two basic routes have been suggested: that it infected humans catching and eating the natural host or that it came from use of contaminated cells in the preparation of one or more vaccines for protecting humans against other viruses. The former scenario would not be a unique human intervention as it could happen to any carnivore species. The second, being a human activity in medical treatment, definitely is a human intervention. The second area of human influence is in the worldwide spread of the viruses, which has been exacerbated by human activities such as international travel, medical treatments such as blood transfusions, and social activities such as intravenous drug uptake.

C. Examples of Impact of Human Behavior on Plant Viruses

Three major features of crop agriculture, plant breeding, plant distribution, and growing plants as monocultures, have influenced virus–plant coevolution. Over the last 100 or so years there have been plant breeding programs directed to provide resistance against viruses. The forms of natural resistance used in breeding programs are described in Hull (2001). Many of the resistance genes are monogenic and dominant and thus can be considered to be subject to the constraints of Flor's gene-for-gene hypothesis (Flor, 1971). This hypothesis proposes that for resistance to occur, complementary pairs of dominant genes, one in the host and the other in the pathogen (the virulence locus), are required. Mutations in the virulence locus of the pathogen can overcome the resistance trait of the host gene, and thus deployment of the resistance gene selects for pathogenic mutations. Examples of this occurring among plant viruses are reviewed by Hull (2001).

The development of modern agriculture has resulted in the movement of crop species out of their centers of origin and diversification to wherever they can be grown worldwide. It has been suggested for several viruses that they have geographical centers of origin, which generally correspond to the centers of origin of their main hosts. For example, *Citrus tristeza virus* and its citrus hosts have their center of origin in south Asia (Lovisolo, 1993); *Sugarcane mosaic virus* and sugarcane have their center of origin in Indonesia (Lovisolo,

1989–1990). These centers of origin probably refer to a recent geological event because living terrestrial organisms changed distribution in the course of the different eras. For instance, most geological evidence shows that the North Atlantic land bridge, which connected the floras of Europe and North America, was severed about 50 mya (Milne and Abbott, 2002).

The widespread distribution of crop plants has led to susceptible species being exposed to indigenous viruses to which they have not been previously adapted. There are many examples of this, including the movement of cacao from South America to west Africa where it became infected by Cacao swollen shoot virus, an indigenous virus of some local forest trees (e.g., *Cola* spp.). The virus, which is symptomless in indigenous trees, causes a devastating disease of the introduced cacao.

The development of modern agriculture has also spread viruses, especially of vegetatively propagated crops such as banana and potato, to new environments where they could come into contact with other viruses. There is an increasing number of examples of new viruses arising from recombination between viruses. A good recent example is shown in the severe outbreak of cassava mosaic disease in east Africa. Analysis of the virus causing this disease in Uganda revealed that it was the result of natural inter- and intraspecies pseudorecombinations between the geminiviruses *African cassava mosaic virus* and *East African cassava mosaic virus* (Pita *et al*., 2001). It is not known what brought these two viruses together but, like cacao, cassava originated in South America where these two viruses do not exist. A further study on cassava mosaic geminiviruses showed that coadaptation exists between the virus and the local whitefly vector populations (Maruthi *et al*., 2002).

To enhance food production, many crops are now grown as extensive monocultures. It is quite possible that this has had an influence on the interactions among viruses, their hosts, and their vectors. The intensification of rice cultivation in Asia during the "green revolution" was associated with an increase in the rice green leafhopper *Nephotettix virescens* and the tungro virus disease that it transmits (see Anjaneyulu *et al*., 1995). Tungro disease is caused by a complex of two viruses: the sequivirus *Rice tungro spherical virus* (RTSV), which is transmitted by *N. virescens* but gives few symptoms in rice, and a member of the *Caulimoviridae*, RTBV, which gives the severe symptoms of the disease but is not leafhopper transmitted on its own. In the tungro complex, RTBV gives the symptoms and RTSV mediates the transmission of both viruses. No natural vector is known for RTBV, but

the related badnaviruses are primarily mealybug transmitted. Questions arise as to what are the natural hosts of these two viruses and how they came together in the complex. It is possible that the intervention of humans was a major factor in the generation of this disease complex.

D. Discussion

Viruses are widespread in many living organisms and probably had much diversified before the appearance of humans. However, as shown earlier, human activity has had, and is still having, a significant impact on the coevolution of viruses, their hosts, and vectors.

As discussed in Section III,B, during the course of the long coevolution with their hosts and vectors, viruses have, in many cases, achieved a form of equilibrium. The activities of humans are breaking this equilibrium continually and, because of the great potential of variability of viruses (see Domingo and Holland, 1994), create the conditions for the appearance of new virus–vector–host interactions.

VI. Discussion and Further Perspectives

This review explored the evidence for coevolution of viruses, their hosts, and their vectors. While there are reasonable observations on recent events, especially those associated with activities of humans, longer-term events are speculative. However, several facets are becoming apparent.

1. Viruses probably evolved early in time but, as they need complete cells for replication, it is not possible to know if RNA or DNA viral genomes evolved first. Also, it is not possible to predict whether early viruses resemble any of the current ones or whether virus evolution relates to the major evolutionary events of their hosts.

2. It is very likely that arthropods played an important role in the coevolution of viruses and hosts, both as initial hosts and as vectors. The interplay of arthropod interactions, especially with plants, is significant in the development of coevolution of viruses.

3. The comparative analyses of viral genomes and the increasing understanding of how viruses function in their hosts have led to the suggestion that viruses adapt to ecological "niches" within the cell (Hull and Covey, 1996). This adaptation ranges from the acquisition of cassettes to enable the virus to exist in the host to refinements of

replication and expression so that it causes minimum damage to the host. Thus, continual change in the detailed biochemistry and structures within the cell could lead to continual selection on viral quasispecies to give the fittest form for that niche. It is not known where the adaptation cassettes come from, but Hull (2001) suggested that the virus acquires them from the host and they become adapted to optimize the virus infection cycle.

4. As well as viruses adapting to their hosts there are examples of hosts adapting to viruses. The retention of integrated viral DNA sequences in the host genome indicates that these sequences are advantageous to the host. As noted in Section III,D,5, it is suggested that these sequences may give the host some form of resistance to the virus. The widespread presence of "dead" retrotransposon sequences in the genomes of many plants and animals might indicate that integrated sequences also have other functions in the maintenance and expression of the host genome.

This analysis shows that interactions among viruses, their hosts, and vectors are dynamic and that there is continuous coevolution. The application of new technologies and the awareness of scientists working in disciplines such as paleontology and molecular biology are likely to throw further light on these interactions.

NOTE ADDED IN PROOF: Of considerable relevance to the coevolution of viruses with their hosts is the recent report of virus infection inducing plant host genome DNA rearrangements (Kovalchuck *et al.,* 2003).

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