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Complexity and Versatility of the Transcriptional Response to cAMP

V. DELMAS, C. A. MOLINA, E. LALLI, R. DE GROOT, N. S. FOULKES, D. MASQUILIER, and P. SASSONE-CORSI

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Laboratoire de Génétique Moléculaire des Eucaryotes, CNRS, U184 INSERM, Institut de Chimie Biologique, Faculté de Médécine 11, rue Humann, 67085 Strasbourg, France

1 Introduction

The regulation of gene expression by specific signal transduction pathways is closely connected to the cell phenotype. The response elicited by a given transduction pathway varies according to the cell type. The finding that most of the known nuclear oncogenes encode proteins involved in the regulation of gene expression inspired the concept that the aberrant expression of some key genes could cause cellular transformation or altered proliferation (Lewin 1991). The study, and ultimately the understanding, of these processes will hopefully help us to unravel the profound changes that cause cancer and by the same token the physiology of normal growth.

An important step toward the comprehension of how the function of transcription factors can be modulated has been the discovery that many constitute final targets of specific signal transduction pathways, activated intracellularly by various signals at the cell surface. The two major signal transduction systems are those including cAMP and diacylglycerol (DAG) as secondary messengers (Nishizuka 1986). Each pathway is also characterized by specific protein kinases [protein kinase A (PKA) and protein kinase C (PKC), respectively] and its ultimate target DNA control element, the cAMP-responsive element (CRE) and the TPA-responsive element (TRE). Although initially characterized as distinct systems, accumulating evidence points toward extensive cross-talk between these two pathways (Cambier et al. 1987; Yoshimasa et al. 1987). Here we focus primarily on the targets of the cAMP-mediated transduction response.

2 The PKA Signal Transduction Pathway

Intracellular levels of cAMP are regulated primarily by adenylyl cyclase. This enzyme is in turn modulated by various extracellular stimuli mediated by receptors and their interaction with G proteins (McKnight et al. 1988). The binding of a specific ligand to a receptor results in the activation or inhibition of the cAMP-dependent pathway, ultimately affecting the transcriptional regulation of various genes through distinct promoter-responsive sites (Montmayeur and Borrelli 1991). Increased cAMP levels directly affect the function of the tetrameric PKA complex (Krebs and Beavo 1979). Binding of cAMP to two PKA-regulatory subunits releases the catalytic subunits enabling them to phosphorylate target proteins. A number of isoforms for both the regulatory and catalytic subunits have been identified, suggesting a further level of complexity in this response (McKnight et al. 1988). In the nucleus the phosphorylation state of transcription factors and related proteins appears directly to modulate their function and thus the expression of cAMP-inducible genes.

3 Promoter Sites of cAMP-Inducible Genes

3.1 The cAMP-Responsive Element

A promoter element that mediates the response to increased levels of intracellular cAMP is the CRE (Comb et al. 1986; Andrisani et al. 1987; Delegeane et al. 1987; Sassone-Corsi 1988). A consensus CRE site constitutes an 8-bp palindromic sequence (TGACGTCA). Several genes which are regulated by a variety of endocrinological stimuli contain similar sequences in their promoter regions although at different positions. A comparison of the CRE sequences identified to date shows that the 5' half of the palindrome, TGACG, is the best conserved, whereas the 3' TCA motif is less constant. The binding site specificity appears to require 18–20 bp, since the five or so bases flanking the core consensus have been shown to dictate, in some cases, the permissivity of transcriptional activation (Deutsch et al. 1988). In many genes the CRE sequence is located in the first 200 bp upstream from the cap site. In most cases there is only one CRE element per promoter, although there are notable exceptions. The promoter of the α-chorionic gonadotropin gene, for instance, contains two identical, canonical CREs in tandem, between positions –117 and –142 (Delegeane et al. 1987). The promoter of the pituitary-specific transcription factor GHF-1/Pit-1, on the other hand, contains two different CREs between positions –200 and –150, which are separated by a 40-bp spacer (McCormick et al. 1990). The proto-oncogene c-*fos* contains a powerful CRE at position –60 (Sassone-Corsi et al. 1988a), but other CRE-like sequences are also present within the gene regulatory region (Berkowitz et al. 1989); however their precise function has yet to be determined.

The CRE consensus sequence also appears in the context of other promoter elements. These include the ATF sites in the early promoters of adenovirus (Lin and Green 1988; Sassone-Corsi 1988), the 21-bp tax-dependent enhancer of the human T-cell leukemia virus (HTLV-I) virus long terminal repeat (LTR; Yoshimura et al. 1990), and the X-box motif associated with MHC class II genes (Liou et al. 1988).

3.2 Non-CRE Sites

Recently an additional sequence responsive to increased cAMP levels has been described in the promoter of the steroid hydroxylase gene CYP17 (Zanger et al. 1991). Although no information is available yet on the regulatory proteins which bind to this novel element, it is tempting to speculate that more than one class of sequence element can mediate the response to cAMP,

possibly in a development- or cell-specific manner. In further support of this notion, another non-CRE element which also mediates cAMP induction is the AP-2 recognition site (Imagawa et al. 1987).

4 Structure of the Nuclear Effectors of the PKA Pathway

An important step toward the understanding of cAMP-regulated gene transcription has been made with the cloning of cDNAs encoding CRE binding proteins, thereby allowing studies on the precise structure-function relationship of these factors. At least ten CRE binding factor cDNAs have been isolated. They were obtained by screening a variety of cDNA expression libraries with CRE and ATF sites, the HTLV-I LTR 21-bp repeat, and the MHC class II X-box sequences. They all belong to the basic region/leucine-zipper (b-Zip) family of proteins. It has been demonstrated that, as in the case of Fos, Jun and C/EBP, the leucine zipper is responsible for the dimerization of the protein, and that dimerization is a prerequisite for DNA binding. The model by Vinson et al. (1989) suggests the presence of a *bipartite* DNA binding domain, as dimerization ensures the correct orientation of the adjacent basic regions in order to allow their optimal contact with the recognition sequence. It has been defined that the basic region, 50% rich in lysine and arginine residues, is infact divided into two subdomains containing clusters of basic residues separated by a "spacer" of alanines, conserved among all leucine zipper transcription factors. In this model these two regions recognize the two halves of the palindromic recognition sequence. The positively charged amino acids in the basic region lie on one face of the two helices of a helix-bend-helix structure. The two positively charged α-helices lie in the major groove of the DNA helix positioned so that the positive charges are in contact with the negative charges of the phosphate backbone (Vinson et al. 1989).

All these different proteins are highly homologous in their b-Zip region, while they diverge in the other parts of the proteins. Based on common regions of homology, these factors can be divided into subfamilies. For example, CREB, ATF1, and CREM share extensive regions of homology (Foulkes et al. 1991a; Hai et al. 1989) but are clearly distinct from ATFa and CRE-BP1, which constitute another subfamily (Ivashkiv et al. 1990; Gaire et al. 1990).

The b-Zip is responsible for DNA binding as well as for dimerization of the proteins. The different factors are able to heterodimerize with each other but only in certain combinations. A "dimerization code" exists that seems to be a property of the leucine zipper structure of each factor. For example, ATF1 can dimerize with CREB but not with CREBP1 or ATF3, and

CREBP1 can heterodimerize with ATF3 but not with CREB (Hurst et al. 1991; Hai et al. 1989; Hoeffler et al. 1991). Although most of these heterodimers have not yet been identified in vivo, these observations suggest that part of the complexity of CRE-dependent regulation is accounted for by the large number of homo- and heterodimers combinations possible.

5 Model of Transcriptional Activation

5.1 The CREB Gene

The identification and cloning of the CRE binding protein (CREB) gene opened the way for a molecular analysis of the trans-activation phenomenom. The CREB cDNAs have been cloned from human placenta and rat brain libraries (Hoeffler et al. 1988; Gonzales et al. 1989). CREB is ubiquitously expressed, suggesting a housekeeping role for this factor.

5.2 Activation by Phosphorylation

An important insight into the molecular mechanisms by which the transcription of CRE-containing genes is induced, came from experiments demonstrating that upon activation of the adenylyl cyclase pathway, a serine residue at position 133 of CREB is phosphorylated by PKA (Fig. 1; Gonzales and Montminy 1989). The phosphorylation appears indispensable for activation, and the phosphoserine cannot be substituted by other negatively charged residues (Yamamoto et al. 1988; Gonzales and Montminy 1989; Lee et al. 1990). Whether phosphorylation by PKA modulates DNA binding by CREB is a slightly controversial point. Indeed, Yamamoto et al. (1988) indicate that PKA-mediated phosphorylation of CREB does not affect DNA binding. By contrast, Nichols et al. (1992) have reported that phosphorylation of CREB by PKA causes a modest increase in binding to high-affinity CRE sites but a stronger enhancement in binding to low-affinity CREs. However, the major effect of phosphorylation rather seems to occur at the level of the trans-activation function of CREB. It has been proposed that this could happen by inducing a conformational change of the protein (Gonzalez et al. 1991). However, in contrast to this hypothesis, recent studies by Leonard et al. (1992) have shown that CREB can be a very potent activator in the absence of phosphorylation in the pancreatic islet cell line Tu6. The mechanism of this phosphorylation-independent activity remains to be determined. Interestingly, alternative signal transduction pathways can also induce phosphorylation of serine 133. In PC12 cells, increases in the levels

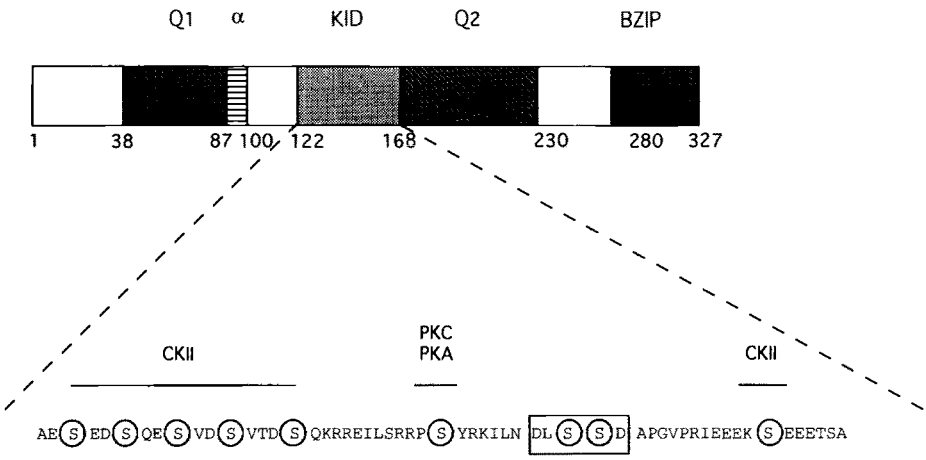

Fig. 1. Structure of transcriptional activator CREB and localization of the phosphorylated residues. The detailed structure of the kinase-inducible domain (*KID*) and the serine residues (*circles*) phosphorylated by the indicated kinases are shown: *PKA*, protein kinase A; *PKC*, protein kinase C; *CKII*, casein kinase II. *Shaded boxes*, KID , α-region (α), and flanking glutamine-rich domains (*Q*). *BZIP*, The basic domain and leucine zipper of the DNA binding region. Numbers of amino acids are given beneath each domain

of intracellular Ca^{2+} by membrane depolarization cause phosphorylation of serine 133 and a concomitant induction of c-*fos* gene expression mediated by a CRE in the c-*fos* promoter (Sassone-Corsi et al. 1988a; Sheng et al. 1990, 1991). CREB mutants lacking serine 133 were unable to activate c-*fos* transcription (Sheng et al. 1991). Although Ca^{2+} calmodulin-dependent (CAM) kinases were shown to be able to phosphorylate serine 133 in vitro (Sheng et al. 1991; Dash et al. 1991), their role in vivo remains unclear, since PKA seems to be necessary for c-*fos* induction by Ca^{2+} influx in PC12 cells (Ginty et al. 1991). In addition, CREB is also phosphorylated upon stimulation by TGFβ1, although the target residue remains to be determined (Kramer et al. 1991).

Recent experiments by Hagiwara et al. (1992) propose a mechanism to explain the attenuation of CREB activity following induction by forskolin. Their results indicate that after the initial burst of phosphorylation in response to cAMP, CREB is dephosphorylated in vivo by protein phosphatase-1 (PP-1), and transcription of the somatostatin gene is correspondingly reduced. However, Nichols et al. (1992) show that both PP-1 and PP-2A can dephosphorylate CREB in vitro, resulting in a decreased binding to low-affinity CRE sites in vitro. Therefore, the precise role of PP-2A in the dephosphorylation of CREB remains to be determined.

The structure of the transcriptional activation domain of CREB includes more than the phosphoacceptor region (see Fig. 1). Serine 133 is located in a region of about 50 amino acids containing an abundance of phosphorylated serines and acidic residues, the phosphorylation box (P-Box) or kinase inducible domain (KID), which was shown to be essential for *trans*-activation by CREB (Lee et al. 1990; Gonzalez et al. 1991). Although phosphorylation of serine 133 appears indispensable for activation by CREB, it is not sufficient for full activity. An acidic region just downstream of serine 133 (140-DLSSD) was shown to be important for CREB function (Lee et al. 1990; Gonzalez et al. 1991). In addition, deletion of a region called α2, containing several sites that can be phosphorylated by CKII in vitro, caused a decrease in CREB activity, although differences in the magnitude of this decrease were reported (Lee et al. 1990; Gonzalez et al. 1991).

Interestingly, two other CRE binding factors have been reported to be activated by PKA. ATF1 was shown to activate transcription after cotransfection of the catalytic subunit of PKA (Rehfuss et al. 1991; Flint et al. 1991). Although ATF1 can be phosphorylated by PKA in vitro, in vivo phosphorylation has yet to be demonstrated. The recently described activator isoform of CREM, CREMτ, can also mediate cAMP-induced transcription (Foulkes et al. 1992; see below). CREMτ is phosphorylated by PKA in vitro as well as in vivo on serine 117, the counterpart of serine 133 in CREB (deGroot et al. 1993).

5.3 The Roles of the Glutamine-Rich Domains

Flanking the P-box are two regions in which there are about three-times more glutamine residues than in the remainder of the protein (see Fig. 1). Glutamine-rich domains have been characterized in other factors, such as AP-2 and Sp1 (Williams et al. 1988; Courey and Tjian 1989) as transcriptional activation domains. The significance of the first glutamine-rich domain (Q1) is not completely clear, since it was reported to enhance CREB activity by Gonzalez et al. (1991) while Lee et al. (1990) failed to find an effect when they deleted this region. However, this apparent contradiction might be caused by the different CREB isoforms studied by these two groups, since Gonzalez et al. studied CREBα/341, while Lee et al. have used CREBD/327. Interestingly, two different CREM isoforms containing either Q1 (τ1) or Q2 (τ2) are both transcriptional activators (Fig. 2). The Q2 domains appears to confer a slightly higher activation potential than the Q1 domain (Laoide et al. 1993). These results demonstrate that the Q1 and Q2 regions probably function in an additive manner to generate the full activation potential of CREMτ.

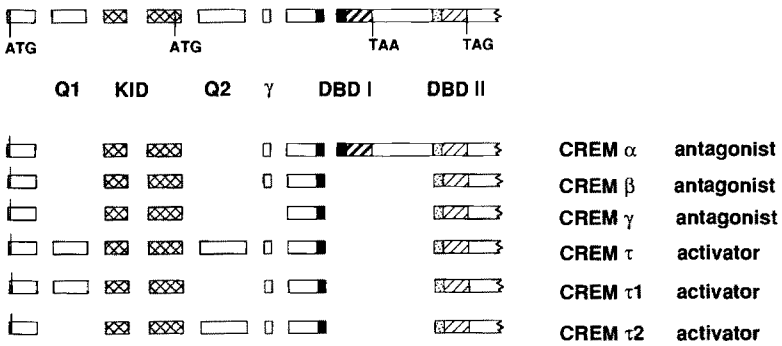

Fig. 2. CREM multiexonic structure generates isoforms with different functions. Schematic representation of CREM exon structure and transcripts. *Top row*, the various exons derermine the functional domains of the proteins (Laoide et al. 1993). *Q1, Q2*, glutamine-rich domains; *KID*, kinase-inducible domain, γ, a 12 amino acid domain lacking in the CREMγ isoform and which has no counterpart in CREB; *DBDI, DBDII*, the two alternative DNA binding domains. *Hatched boxes*, the leucine zipper portion of the two DNA binding domains; *solid black boxes*, the basic region of DBDI and the shared 5' portion of the basic domain of DBDII. The position of the initiation (ATG) and termination codons (TAA, TAG) are indicated. The position of the alternative ATG used to generate S-CREM (Delmas et al. 1992) is also shown. Beneath is also shown the exon composition of the various CREM isoforms

The current model to explain the activation of CREB suggests that upon phosphorylation of serine 133 by PKA, a conformational change is induced, which leads to exposure of the glutamine-rich activation domains (Gonzalez et al. 1991). The other regions which were identified as being important for CREB function might be involved in correctly spacing the phosphorylation site with respect to the glutamine-rich domains. Verification of this model awaits determination of the crystal structure of both unphosphorylated as well as phosphorylated CREB.

6 Molecular Basis of Negative Regulation: The CREM Gene

The discovery of the cAMP-responsive element modulator (CREM) gene opened a new dimension in the study of transcriptional response to cAMP (Foulkes et al. 1991a). This is due to the remarkable genomic structure of the CREM gene, which offers clues to the understanding of the generation of functional diversity in transcription factors. CREM is also the first CRE binding protein with antagonistic function.

6.1 A Remarkable Genomic Structure and Cell-Specific Expression

The CREM gene was isolated from a mouse pituitary cDNA library screened at low stringency with oligonucleotides corresponding to the leucine-zipper and basic region of CREB. The logic behind this approach is that the adenylyl cyclase pathway plays an important role in the modulation of the hormonal regulation in the pituitary gland. The most striking feature about the CREM cDNA is the presence of two DNA binding domains. The first is complete and contains a leucine zipper and basic region very similar to CREB; the second is located in the 3' untranslated region of the gene, out of phase with the main coding region, and contains a half basic region and a leucine zipper more divergent from CREB. Various mRNA isoforms were identified that appear to be obtained by differential cell-specific splicing (Fig. 2). Alternative usage of the two DNA binding domains was demonstrated in various tissues and cell types, where quite different patterns of expression were found (Foulkes et al. 1991a). This strongly contrasts with CREB and ATFs expression which is ubiquitous (Hai et al. 1989; Habener 1990), suggesting their roles as constitutive regulators.

CREM expression appears to be finely regulated, both transcriptionally and posttranscriptionally. In fact, not only cell- and tissue-specific expression is observed, but also the production of various isoforms. Four major isoforms have been characterized, of which three act as repressors and one as a powerful activator. The three antagonistic products were the first to be described (Foulkes et al. 1991a). These isoforms revealed alternative usage of the two DNA binding domains (α and β isoforms), as well as a small deletion of 12 amino acids (γ isoform; Fig. 2). The potential of even more complexity of CREM regulation is hinted at by the possible usage of alternative poly(A) addition sites and by the presence of ten AUUUA sequences in the 3' untranslated region, elements thought to be involved in mRNA instability (see Fig. 3; Shaw and Kamen 1986). The strict cell- and tissue-specific expression of CREM is indicative of a pivotal function in the regulation of cell-specific cAMP response. This suggests that CREM occupies a central control point in the pituitary, since it is known that the physiology of this gland is finely regulated by a multiplicity of hormones whose coupled signal transduction pathways involve adenylate cyclase. Interestingly, other well-described examples of cell-specific splicing include the genes encoding neuronal peptides and hormones in brain and pituitary cells (Leff et al. 1986). It appears clear, thus, that cell-specific splicing is a crucial mechanism of CREM regulation, which modulates the DNA binding specificity and activity of the final CREM products.

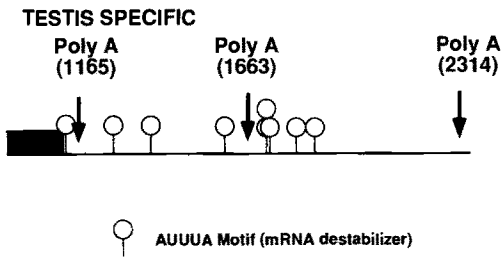

Fig. 3. Schematic representation of the 3' untranslated region of the CREM gene (Foulkes et al. 1991a). Three alternative polyadenylation signals are used (*PolyA*); their position within the CREM cDNA is indicated. The farthest upstream PolyA site is used predominantly in male germ cells. In the region there are ten destabilizer sequences AUUUA (*open circles*; Shaw and Kamen 1986)

6.2 Antagonists and Activators from the Same Gene

The first cDNA clones which were characterized from the CREM gene encode antagonists of cAMP-induced transcription (Foulkes et al. 1991a). The CREM antagonists share extensive homology with CREB, but they lack two glutamine-rich domains, which have been shown to be necessary for transcriptional activation in CREB (Gonzalez et al. 1991). Interestingly, the CREM gene also encodes an activator of transcription (Foulkes et al. 1992). In the adult testis, an isoform CREMτ has been identified which resembles in structure one of the antagonist forms (CREMβ) but includes two exons that encode two glutamine-rich domains. As mentioned before, this form has been demonstrated to transactivate transcription from a CRE site, as well as the CREM isoforms containing either Q1 or Q2 glutamine-rich domains (Laoide et al. 1993).

The CREM proteins specifically recognize CREs and show the same binding properties as CREB. This is not surprising, considering the high homology in the DNA binding domains between these proteins. CREM proteins containing either DNA binding domain I or II heterodimerize with CREB (Foulkes et. al. 1991a; Laoide et al. 1993), although it appears that CREMα-CREB heterodimer formation is more favored than CREMβ-CREB. These notions suggest that CREM proteins might occupy CRE sites as CREM dimers or as CREM-CREB heterodimers, thus generating complexes with altered transcriptional functions. Infact CREM products act by impairing CRE-mediated transcription, and as such are considered as antagonists of cAMP-induced expression. In transfection experiments, using CRE reporter plasmids, it was demonstrated that CREM antagonists block the transcriptional activation obtained by the joint action of CREB or CREMτ and the catalytic subunit of the cAMP-dependent PKA (Mellon et

al. 1989). These observations strongly support the notion that CREM proteins negatively modulate CRE promoter elements in vivo. An important question is how CREM proteins work. The two most likely hypotheses are as follows. According to the first scenario, CREM proteins dimerize and bind to CRE sites. Downregulation is achieved by the occupation of these sites, which are unavailable for CREB or CREMτ. Similarly, if CREB is already bound, CREM proteins might squelch them because of their possible higher affinity for a specific site. According to the second model, CREM proteins are able to dimerize with CREB to generate non-functional heterodimers. Negative regulation is achieved by titrating active CREB molecules, and CREM proteins could act as activator traps. Since both CREM dimers and CREB-CREM heterodimers bind to CRE sites, both hypotheses are justified and both mechanisms may operate. The CREM mRNA isoforms are a graphic illustration of how alternative splicing can modulate the function of a transcription factor in a tissue- and developmental-specific manner (Foulkes and Sassone-Corsi 1992).

6.3 Alternative Translation Initiation: Another Way to Generate a Repressor

We have also shown that by alternative usage of translation initiation sites, a single CREM mRNA generates both an activator and a repressor (Delmas et al. 1992). The use of an internal AUG in the CREMτ transcript generates S-CREM, a protein that acts as a powerful repressor of cAMP-induced transcription. It is puzzling that CREM, which already makes extensive use of differential splicing to generate both an activator and antagonists (Fig. 2), utilizes an additional mechanism to generate factors with opposite functions. It is important to note, however, that S-CREM is distinct from the antagonist CREM forms generated by alternative splicing. It does not contain the phosphoacceptor sites (P-box), but instead contains a single glutamine-rich domain. This is in contrast to the CREMα/β/γ antagonists, which contain the P-box but no glutamine-rich domains (Foulkes et al. 1991a). It is reasonable to hypothesize that the structural differences present between these various downregulators have functional significance, and that these may interact differently with other components of the transcriptional machinery. In addition, the differential presence of the P-box among these various CREM downregulators, could suggest that some of them might be modulated in their function by phosphorylation.

Internal initiation has been described for others genes, for example the oncogenes *int*2 (Acland et al. 1991), *pim*-1(Saris et al. 1991) and the androgen receptor *Tfm* (Gaspar et al. 1991). Interestingly, alternative initiation

has been described for another leucine-zipper regulatory protein, the Liver Activator Protein (LAP; Descombes et al. 1991). In LAP, an internal AUG is used to produce LIP, a repressor protein. The major difference between LAP and CREM is that LAP is an intronless gene, whereas CREM has a multiexonic structure (Laoide et al. 1993; and in preparation), which, by differential splicing, is already the basis for extensive functional modulation.

6.4 Downregulation of Nuclear Oncogenes

The oncogene c-*fos* is induced by the activation of adenylyl cyclase through several CRE sites located in different regions of the promoter, although the contribution of the site at position –60 to cAMP-inducibility is major (Sassone-Corsi et al. 1988a; Fisch et al. 1989; Berkowitz et al. 1989). cAMP induction of c-*fos* is followed by a rapid decrease in transcriptional rate, reminiscent of the downregulation observed after serum stimulation (Bravo et al. 1987; Verma and Sassone-Corsi 1987). Fos protein is known to be responsible for the *fos* downregulation after serum stimulation by a mechanism of negative feedback autoregulation exerted on the serum responsive element centered at position –300 of the promoter (Sassone-Corsi et al. 1988b). Fos failed in downregulating the cAMP-induced c-*fos* transcription, making clear that the decrease in transcriptional rate after various stimulatory events could be mediated by different effectors (Foulkes et al. 1991b). It has been shown, in fact, that CREM products are able to downregulate c-*fos* cAMP-induced transcription, whereas are not involved in the downregulation after serum induction. It is possible that this mechanism might have a physiological significance; indeed, the downregulation which follows the induction of c-*fos* expression in the supraoptic nucleus in the brain after osmotic stimulation is paralleled by a sharp increase in CREM antagonists (Mellström et al. 1993). Experiments involving antisense CREM indicated that endogenous CREM could be blocked, thus allowing enhanced cAMP-inducibility and an increased basal c-*fos* transcription level (Foulkes et al. 1991b). Similar experiments conducted on the c-*jun* gene, which is naturally downregulated by cAMP treatment of cultured cells, indicate that CREM is likely to be a molecular effector of this physiological event (E. Benusiglio, personal communication).

7 *Trans*-Activation by Viral Products via the CRE/ATF Binding Proteins

7.1 The ATF Family

Several cDNAs encoding different polypeptides which bind to an ATF site have been cloned and partially characterized (Hai et al. 1989). Screening of a HeLa cell cDNA library revealed the presence of a large family of genes encoding proteins with high homology in the DNA binding domain. Two ATF genes have been characterized in more detail, ATF1 and ATF2. ATF2 is homologous to CRE-BP1, a gene cloned from a brain cDNA library (Maekawa et al. 1989) and to XBP-1 (Kara et al. 1990), cloned from a B-cell specific cDNA library. Despite the high degree of homology among ATF gene products, their combinatorial association is not always possible. Thus, while ATF2 and ATF3 form heterodimers, ATF1 does not dimerize with ATF2 or ATF3. Another noteworthy ATF gene is ATF5, which contains a DNA binding domain with high homology to Fos, suggesting, as in the case of ATF2, possible heterodimerization with nuclear targets of the PKC pathway. All ATF genes characterized to date (Hai et al. 1989) show ubiquitous expression, suggesting that their intracellular levels are unlikely to be major determinants of their activity. Also as with CREB, the ones which have been characterized have trans-regulatory potential and behave as positive activators (Liu and Green 1990).

7.2 Viral Induction via ATF/CRE Sequences

The identification of the CRE as the element necessary for transcriptional activation by elevated cAMP levels was paralleled by the characterization of a similar sequence (the ATF site) to be important in several early promoters of adenovirus (Lin and Green 1988; Sassone-Corsi 1988). ATF sites are responsible for the cAMP-inducibility of some of these promoters. In addition, it was shown that the ATF site in the E4 promoter was crucial for transcriptional activation by the adenovirus E1A protein as well as capable of conferring E1A-inducibility to heterologous promoters (Lee et al. 1989). Interestingly, a number of CRE-containing promoters of cellular genes are inducible by both cAMP and E1A. Recent experiments by several laboratories have shown that E1A-inducibility of CRE/ATF sites is mediated by a specific member of the CRE binding factor family, ATF2 (CREB2, CRE-BP1; Flint and Jones 1991; Lillie and Green 1989; Liu and Green 1990). By fusing specific domains of ATF2 to the DNA binding domain of GAL4 or c-*myb* it was shown that the N-terminal region of ATF2, which contains a

putative zinc-finger structure, is both necessary and sufficient for activation by E1A (Flint and Jones 1991; Liu and Green 1990). Interestingly, the N-terminal region of ATF2 can also mediate transcriptional activation by the product of the retinoblastoma gene (Rb; Kim et al. 1992). Substitution of the cysteine residues in the finger structure strongly decreases activation by E1A (Flint and Jones 1991). Studies using GAL4-E1A fusion proteins have also shown that E1A contains a powerful transcription activation domain (Lillie and Green 1989). Therefore, the current model for activation of CRE sites by E1A involves ATF2 as a promoter-bound receptor for the E1A protein, thereby bringing the activation domain of E1A in the proximity of the transcription initiation complex. However, since no direct protein contacts have been demonstrated between ATF2 and E1A, an intermediate factor might act as an adaptor between the two proteins.

Viral induction via CRE sequences does not seem to be limited to the products of adenoviruses. Du and Maniatis (1992) have shown that the element responsible for viral induction of the human interferon-β (HuIFN-β) gene overlaps with a CRE. Mutations in the CRE that diminished binding by ATF2 in vitro also decrease viral induction in vivo. In addition, multiple copies of the virus-inducible element confer both virus and cAMP inducibility to a heterologous promoter. However, the CRE was not sufficient for viral inducibility of HuIFN-β, and additional flanking sequences have been shown to be indispensable.

Another example of viral activation operating through CREs comes from studies on the induction of the HTLV-I LTR of human T-cell leukemia virus (HTLV-I) by the HTLV-I *tax* protein. It was shown that this induction is mediated by three 21-bp repeats present in the LTR (Tan et al. 1989). All three repeats contain a CRE consensus sequence, which are essential for LTR induction by *tax*, and which are able to bind CREB and CREM in vitro (Laoide et al. 1993; Beimling and Moelling 1992). As in the previous case of the IFN-β gene, additional flanking sequences are also required for *tax* induction of the LTR (Fujisawa et al. 1989). Moreover, the *trans*-activation potential of neither CREB, ATF1, nor ATF2 (fused to a GAL4 DNA binding domain) was activated by *tax* in transient cotransfections (Flint and Jones 1991). Thus, the precise mechanism of induction remains unclear.

The most striking example of viral induction through a CRE comes from recent experiments by Maguire et al. (1991). They have shown that induction of the hepatitis B virus (HBV) enhancer element is mediated by a CRE-like sequence. This sequence fails to bind either CREB or ATF2 in vitro. However, when the HBV activator pX was included in the reactions, both CREB and ATF2 efficiently bound to the HBV CRE. Direct protein-protein interactions between pX and CREB or ATF2 have also been demonstrated. Thus, the ability of pX to interact with cellular transcription factors

alters their DNA binding specificity, and may ultimately modify the repertoire of genes expressed during viral infection.

8 Physiological Importance of CRE-Binding Proteins

Although the results described above clearly demonstrate that CRE binding factors are important for cAMP-mediated transcriptional regulation in cultured cells, not much has been demonstrated about the specific physiological roles for these proteins. This is of importance because the crucial role played by the variations in cAMP levels in neuroendocrine regulations.

8.1 CREB Function in Pituitary Development

An interesting first clue for a physiological function of CREB came from experiments using transgenic mice that expressed a CREB mutant which cannot be phosphorylated by PKA (Struthers et al. 1991). Since cAMP serves as a mitogenic signal for the somatotroph cells of the anterior pituitary, the mutant cDNA was placed under the control of the somatotroph-specific promoter of the growth hormone gene. The pituitary glands of transgenic mice expressing this construct were atrophied and were deficient in somatotroph cells. Moreover, the transgenic mice exhibited a dwarf phenotype. No other cell type in the pituitary was influenced by expression of the transgene. These effects might arise from repression of genes involved in proliferation and pituitary-specific gene expression, such as c-*fos* and GHF1/Pit-1, although the expression of these genes was not analyzed in the transgenic animals. It is noteworthy that the block of CREB function by the dominant repressor generated a transgenic phenotype equivalent to the one obtained by targeted cell death of the somatomammotrophs (Borrelli et al. 1989). This could be an indication that CRE binding proteins are likely to have pivotal functions in the normal pituitary development.

8.2 CREM Function in Brain

Changes in intracellular levels of cAMP constitute a major regulatory mechanism of signal transduction in the CNS. To date, several nuclear effectors of this pathway have been characterized, although their functional relevance in brain has been unclear because of their widespread distribution and their constant expression. We have reported the specific and anatomically distinct expression of the antagonist isoforms of the CREM gene in adult rat brain

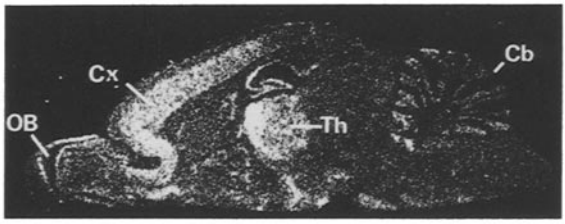

Fig. 4. In situ distribution of CREM transcripts in adult rat brain. Parasagittal sections were hybridized with ^{35}S-labeled antisense riboprobes (Mellström et al. 1993). The probe used for this experiment reveals the distribution of all CREM transcripts. *Cx*, Cerebral cortex; *Cb*, cerebellum; *OB*, olfactory bulb; *Th*, thalmus

and the rapid induction of the α and β isoforms in supraoptic neurons upon physiological stimulation (Mellström et al. 1993). All known CREM isoforms are represented in total brain RNA after PCR amplification (Foulkes et al. 1991a). However, while more quantitative techniques such as RNase protection confirmed the presence of both activators and repressors transcripts, in situ hybridization analysis shows that in neural tissues the antagonist isoforms have a well defined distribution pattern (Fig. 4). In contrast, the activator CREM isoforms, which include the glutamine-rich domains, in common with CREB, have a more diffuse and general distribution. A major point is that CREM differs from the other members of the CRE/ATF family in that specific isoforms are induced upon physiological stimulation. To date, genes of the CRE/ATF class have been described as noninducible (for reviews see Habener 1990; Borrelli et al. 1992). Osmotic stimulation resulted in a differential accumulation of the two antagonist isoforms CREMα and CREMβ, but no change in CREMγ or the activator CREM. Consistent with previous reports (for review see Habener 1990), no induction was observed for the activator CREB.

The induction of several genes in the supraoptic nucleus upon osmotic stimulation has been described previously, including the early response gene c-*fos* which has been shown to undergo a rapid and transient induction (Sherman et al. 1986; Carter and Murphy 1990; Sharp et al. 1991). Since CREM antagonists are able to negatively transregulate the activity of the c-*fos* promoter (Foulkes et al. 1991b), the temporal correlation between the onset of induction of CREMα and -β and the decrease in c-*fos* transcript in supraoptic neurons would suggest a role for CREM antagonists as downregulators of c-*fos* early induction in these neurons. Although our data fall short of unambiguously demonstrating such a role for CREM, they do provide a stimulating basis for future investigations.

A remarkable aspect of the distribution of CREM antagonists in brain is the high level of expression in the anterior thalamic nuclei (Fig. 4). This region, forming a part of the forebrain limbic system, receives input from the hippocampus and the mammillary body of the hypothalamus and projects mainly to the cingulate cortex. This anatomical circuit has been associated with memory and integration of emotions. The significance of CREM expression in the anterior thalamus is unknown, but since this area has been reported to show no induction of early response genes after brain stimulation (Morgan et al. 1987; Sagar et al. 1988; Bullitt 1989), it is tempting to speculate that the high basal expression of CREM antagonists could in part account for this phenomenon. In this respect, it is noteworthy that induction of c-*fos* in the thalamus after peripheral nociceptive or convulsive stimulation occurs in nuclei of the central, midline, and ventral thalamic complexes (Sagar et al. 1988; Bullitt 1989), which in general show a weak hybridization signal for CREM.

A second important observation is the presence of CREM antagonists in almost all the motor nuclei of the brain stem, while sensory nuclei are generally negative. Exceptions to this are the superior olive, which is associated with auditory perceptions, and the mesencephalic trigeminal nucleus, equivalent to the dorsal root ganglia related to propioceptive sensory information, in which CREM transcripts are present. Conversely, CREM expression in motor nuclei includes the somatic motor nuclei – occulomotor, trochlear, abducens, and hypoglossal – as well as the special visceral, trigeminal, and facial nuclei and the general visceral motor nucleus of the vagus. Other positive motor nuclei are the red nucleus, the deep cerebellar nuclei, and the pontine nucleus (Mellström et al. 1993).

Expression of CREM antagonist isoforms in several hypothalamic nuclei associated with homeostatic regulation is also noticeable. Such is the case for magnocellular neurons in the supraoptic hypothalamic nuclei which respond to osmotic stimulation by the differential temporal induction of two of the antagonist isoforms, CREMα and CREMβ. Other functionally related brain areas in which the CREM antagonists are expressed include nuclei involved in visual processing: the suprachiasmatic nucleus, the dorsolateral geniculate nucleus, the lateroposterior thalamic nucleus and the medial terminal nucleus of the accessory optic tract. The latter is supposedly involved in entrainment of endocrine rhythms by light, and fine adjustment of head-eye coordination. Moreover, the presence of CREM in the pineal gland also points to a possible role for CREM in the processing of visual information and the establishment of circadian rhythms.

These findings are a further demonstration of the physiological importance of the CREM gene among the CRE/ATF family of factors. The discovery of the anatomically specific pattern of expression of distinct CREM

isoforms, together with their potential for inducibility, sheds new light on the mechanisms whereby cAMP regulates gene expression in the brain.

8.3 Expression of the CRE Binding Protein During Spermatogenesis

Recently a number of reports have suggested a role for CRE binding proteins in spermatogenesis. This was not unexpected since the metabolism of Sertoli's and Leydig's cells, the somatic cell-types that direct the maturation of germinal cells, is regulated by the pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone, which in turn activate the adenylyl cyclase pathway. The most striking example of differential regulation of CRE binding proteins during spermatogenesis comes from recent studies on CREM (Foulkes et al. 1992). By studying the expression of the CREM gene during spermatogenesis we observed a novel isoform, CREMτ, which is generated by coordinate insertion of two glutamine rich domains in the repressor isoform CREMβ (see Fig. 2). As a consequence of these insertions, CREM behaves as a transcriptional activator. We found an abrupt switch in CREM expression during spermatogenesis. Premeiotic germ cells express only the repressor forms in low amounts, while from the pachytene spermatocyte stage onwards CREMτ is expressed uniquely and in very high amounts. CREMτ RNA is expressed only in spermatocytes and spermatids, while in Sertoli's and Leydig's cells the preswitch pattern is observed (Foulkes et al. 1992). The CREMτ protein is readily detected in spermatids by using CREM-specific antibodies (Fig. 5; Delmas et al. in preparation). Surgically removing the pituitary in vivo caused the reappearance of the preswitch pattern. By reinjection of different hormones into the testis of these animals it was shown that the developmentally regulated switch of CREM is mediated by FSH (Foulkes et al. 1993). Strikingly, the induction of CREMτ does not occur at the transcriptional level but seems to be mediated by FSH-dependent usage of an alternative polyadenylation site, thereby dramatically enhancing transcript stability (see Fig. 3; Foulkes et al. 1993). These results suggest an important role for CREMτ during spermatogenesis.

In the case of CREB the results are somewhat less striking. It was shown by Waeber et al. (1991) that CREB is highly expressed in the haploid round spermatids of rat testis. However, since the antibody used in these studies was raised against a peptide from a region in CREB that is highly homologous to CREM, it cannot be excluded that the nuclear staining results from cross-reaction to CREM. In addition to nuclear localized full-length CREB or CREM, a truncated form of CREB (CREBW), resulting from alternative splicing was abundant in the cytoplasm of germinal cells. Since this form lacks the bZip domain and the nuclear localization signal, it might perform a

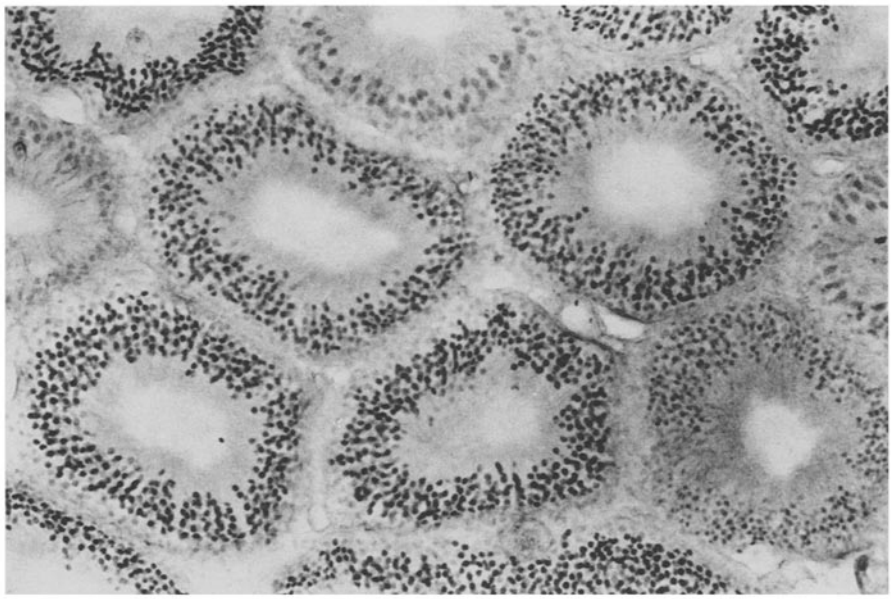

Fig. 5. Peroxidase staining of rat seminiferous tubules showing expression of the CREM protein in the spermatids. The CREM antibody used for this experiment was prepared against a bacterially produced CREMτ protein. Note the differential intensity of the staining in the various tubules, indicating that CREM expression is developmentally regulated

distinct function in the cytoplasm. CREB mRNA was cyclically expressed in Sertoli's cells at stages coinciding with a maximal response of adenylate cyclase to FSH. In addition, Ruppert et al. (1992) demonstrated that the expression CREB and two novel isoforms of CREB, CREBγ and -αγ, was induced in primary spermatocytes after commencement of spermatogenesis. As with CREBW, CREBγ and -αγ encode truncated proteins lacking the bZip domain, the precise role of CREB in spermatogenesis remains to be determined.

9 Cross-Talk at the Nuclear Level

9.1 The PKC-Dependent Pathway

In the PKC-dependent pathway, following the binding of ligands to their cognate receptors, inositol phospholipids are hydrolyzed to generate inositol 1,4,5-triphosphate (IP_3), and DAG, which leads to the activation of PKC (Berridge 1987). In turn, activated PKC phosphorylates several proteins which serve as mediators in the processes of cell proliferation, growth control, and gene regulation.

Transcription factors are among the nuclear targets for PKC transduction pathway (Deutsch et al. 1988; Gilman 1987; Comb et al. 1986; Roesler et al. 1988; Ziff 1990; Binétruy et al. 1991; Pulverer et al. 1991). The nuclear factor AP-1 is phorbol ester-inducible and is constituted by the products encoded by the members of the *jun* gene family, including c-*jun*, *junB*, and *junD* (Bohmann et al. 1987; Angel et al. 1988; Ryder et al. 1988; Lamph et al. 1988; Rauscher et al. 1988; Sassone-Corsi et al. 1998c; Hirai et al. 1989). Jun proteins can homodimerize or heterodimerize with proteins of the Fos family (c–Fos, FosB, Fra-1, and Fra-2; Chiu et al. 1988; Sassone-Corsi et al. 1988c; Zerial et al. 1989; Nishina et al. 1990; Busch and Sassone-Corsi 1990). These dimers bind to TREs present in the promoters of various genes (e.g., metallothionein and collagenase genes), thereby regulating their expression in response to TPA (reviewed in Vogt and Bos 1989; Woodgett 1990).

The *jun* and *fos* genes are members of the immediate early-response class, whose expression is rapidly and transiently induced through intracellular pathways activated by extracellular stimuli (Kruijer et al. 1984; Ryder et al. 1988; Lamph et al. 1988). Functional specificity among the various members of this gene family is likely to be determined by their differential distribution and transcriptional inducibility (Ryder et al. 1988; 1989; Hirai et al. 1989; Naranjo et al. 1991; Mellström et al. 1991). Therefore, the products of these genes act as cell-specific nuclear third messengers, converting cytoplasmic signals into changes in gene expression.

9.2 Cross-Talk Between the PKA and PKC Pathways

Given the many components shared by PKA and PKC pathways, together with the remarkable diversity of agents which can stimulate these pathways, it can be predicted that cross-talk exists between the two signaling systems; indeed, this has been demonstrated in various cases (Cambier et al. 1987; Yoshimasa et al. 1987). Although the difference between TPA and cAMP responsive promoter elements is only one nucleotide (TRE = TGACTCA; CRE = TGACGTCA), they seem to mediate induction only by their respective agonists (Sassone-Corsi et al. 1990). However, this similarity between the nuclear target sequences of the PKA and PKC pathways is striking and suggests possible cross-talk in which CRE binding proteins bind TREs and AP-1 binds CREs.

We have described that the CREM antagonists are capable of negatively modulating the transcriptional activity elicited by Jun/AP-1 (Masquilier and Sassone-Corsi 1992). This phenomenon constitutes a cross-talk between signal transduction pathways at the transcriptional level. Indeed, a canonical TRE can be recognized by two CRE binding proteins, CREB and CREM.

CREM proteins bearing either DNA binding domain are able to bind to TRE sequences. In addition, we observed a dramatic downregulation of the transactivation potential elicited by all Jun proteins (c-Jun, JunB, JunD and v-Jun). There is evidence that the downregulation is likely to be obtained by occupation of the TRE by CREM dimers, since CREM proteins do not heterodimerize with Jun or Fos. Importantly, downregulation is obtained already at a 1:2 ratio of the transfected expression vectors for CREM and Jun, suggesting that CREM binding for the TRE is comparable to Jun. We have found that there are no significant differences between the various CREM proteins in the negative regulation of Jun-mediated transactivation, paralleling the observation that both CREM DNA binding domains confer similar binding activity to the respective CREM proteins (Laoide et al. 1993).

Interestingly, the phosphorylation domain (KID) of CREM is dispensable for the downregulatory function. Infact, CREM truncated proteins which lack the KID region efficiently repress Jun-mediated transactivation. In addition, coexpression of the catalytic subunit of the PKA does not affect the negative regulatory effect of both CREMα and CREMβ. Taking into account these results, and considering that the truncated CREM proteins efficiently bind DNA (Laoide et al. 1993), it seems clear that the negative function of CREM over Jun is almost exclusively due to the occupation of the TRE by the CREM dimer.

One interesting aspect of these results is the fact that transcription factors which could be considered as targets of different signal transduction pathways could affect each others function. It was already shown that Fos-Jun heterodimers bind and activate transcription from CREs (Sassone-Corsi et al. 1990; Hai and Curran 1991). Together with the repression by CREM of the transcriptional activity elicited by Jun/AP1, these observations demonstrate the complexity of the molecular mechanisms of gene regulation and their links with intracellular signal transduction. Thus, there is growing evidence that cross-talk exists at many levels between the two signal transduction pathways.

Interplay may also occur due to promiscuous dimerization among TRE and CRE binding proteins. For example, heterodimerization of ATF2 and Jun switches the specificity of DNA binding with respect to the ATF2 dimer from the CRE sequence to a TRE sequence, thus representing a case of cross-talk between two members of distinct signaling systems (Macgregor et al. 1990; Benbrook and Jones 1990; Ivashkiv et al. 1990; Hai and Curran 1991).

Interestingly, the PKA signal transduction pathway also seems to regulate AP-1 activity through an accessory protein. The characterized inhibitor, IP-1, is itself phosphorylated and thereby inactivated by PKA (Auwerx and Sassone-Corsi 1991). This is reminiscent of the regulation of NFκB by IκB, in which IκB plays an anchoring role (Baeuerle and Baltimore 1988). The

protein, IP-1, is present both in the cytoplasm and nucleus of cells and reduces AP-1 complex formation with DNA in a quick and phosphorylation-dependent fashion. The IP-1 protein appears to be very unstable. IP-1 is regulated by phosphorylation and only in its nonphosphorylated form exerts an inhibitory activity on AP-1 DNA binding. Initial purification attempts showed that the protein to have a molecular weight of approximately 43 kDa. IP-1 itself is subject to a complex regulation. Infact, IP-1 activity was shown to be modulated after activation of several signal transduction pathways, including PKC, PKA and Ca^{2+}/calmodulin-dependent kinase pathways, as well as after serum stimulation of cells (Auwerx and Sassone-Corsi 1992). These data obtained after in vivo stimulation of cells with various agents are in good agreement with previous data showing that (a) IP-1 is inactivated by phosphorylation by PKA in vitro and (b) AP-1 function is activated by the PKA, although c-Jun is not phosphorylated by this kinase (de Groot and Sassone-Corsi 1992). Furthermore, the induced differentiation of cultured cells also appeared to influence IP-1 activity, since treatment of P19 EC cells caused an enhanced AP-1 DNA binding and a correlated reduction of IP-1 activity (Auwerx and Sassone-Corsi 1992).

The AP-2 transcription factor recognizes a site distinct from the TRE or CRE binding sites. The AP-2 site appears to be a target for both the cAMP-dependent and PKC signal transduction pathways and may thus constitute a potential site for cross-talk (Imagawa et al. 1987). However, no direct evidence is available indicating the specificity of AP-2 function. In particular, it is unclear whether AP-2 alone can integrate signals from the two pathways or if it requires additional factors.

10 Conclusion

The large number of CRE binding proteins reveals the complexity of the cellular response to cAMP and possibly suggests the requirements of cell-specificity and potential cross-talk mechanisms with the PKC pathway. The CREM gene constitutes a paradigm that represents another level of complexity. Its modularity of function, which is mediated by alternative and cell-specific splicing events, is an example of the versatility that the cell must accomplish in order to permit normal and regulated cell growth in response to several stimuli. The generation of CREB/CREM-deficent animals by homologous recombination will be an important step in the determination of the precise roles played by the different CRE binding proteins in vivo.

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Suppression of *ras* Oncogene-M diated Transformation

R. SCHÄFER

Contents

Division of Cancer Research, Department of Pathology, University of Zurich, Schmelzbergstrasse 12, CH-8091 Zurich, Switzerland

1 The Function of *ras* in Normal and Transformed Cells

Ras proteins play a fundamental role in the transduction of stimuli from the cellular environment via cell membrane receptors to the nucleus. Both normal and mutated forms of the *ras* gene products have been shown to be involved in the control of proliferation and differentiation. The *ras* genes were originally identified as the transforming genetic elements in the genomes of the Harvey and Kirsten strains of rat sarcoma viruses. The genome of a mammalian cell harbors the Ha-*ras*-1 and Ki-*ras*-2 proto-oncogenes as well as several pseudogenes. A third member of the *ras* gene family, designated N-*ras*, was isolated, for which a retroviral derivative is not yet known (for review see Barbacid 1987; Grand and Owen 1991). Ras proteins belong to the superfamily of small molecular weight GTPases comprising about 50 different members (for review see Bourne et al. 1991). The size of *ras* genes varies from the 4.5 kb of c-Ha-*ras*-1 located on human chromosome 11p15-p15.5 to the 50 kb of c-Ki-*ras*-2 mapped in chromosome 12p12.1-pter. The human N-*ras* gene has been assigned to the short arm of chromosome 1 in band p22-p32. In spite of varying intron structures, the coding sequences of the *ras* genes equally consist of four exons encoding a protein product of 21 kDa, known as $p21^{ras}$. The c-Ki-*ras*-2 gene has two alternative fourth coding exons allowing the synthesis of two isomorphic p21 proteins with a different carboxy-terminal domain. The 85 amino-terminal amino acid residues are identical among mammalian Ras proteins. The following internal 80 amino acid residues share 85% similarity among different *ras* genes, whereas the remaining C-terminal amino acid residues are highly variable except for the terminal four amino acid residues (for review see Barbacid 1987; Grand and Owen 1991).

Cellular oncogenes were identified by transfection of DNA of chemically transformed cells into phenotypically normal recipient cells (Shih et al. 1979; Cooper et al. 1980). Introduction of these genes into preneoplastic murine NIH/3T3 fibroblasts resulted in morphological transformation (focus formation), anchorage-independent proliferation, and tumorigenicity. Later, tumor DNA mediated-transformation of those phenotypically normal indicator cells became a widely used strategy to detect cellular oncogenes. The transforming activity of cellular Ha-*ras*, Ki-*ras*, and N-*ras* genes found in tumors was due to point mutations affecting amino acid codons 12, 13, 59, and 61 (for review see Barbacid 1987; Grand and Owen 1991). Morphologically transformed foci were also induced in NIH/3T3 cells following overexpression of the nonmutated Ha-*ras* gene (Chang et al. 1982). The number of human and experimental tumors screened for *ras* mutations increased significantly, when the laborious NIH/3T3 transfection assays were replaced by direct mutational analysis of the genes using oligonucleotide mismatch

hybridization (Bos et al. 1984) or RNase mismatch cleavage (Winter et al. 1985). The incidence of *ras* gene mutations varied greatly in human tumors. High incidences of mutations were found in carcinomas of the pancreas (90%), the colon (50%), and the lung (30%), in thyroid tumors (50%), and in myeloid leukemia (30%; for review see Bos 1989).

Transforming *ras* genes were not only detected by gene transfer experiments in cultured cells. The critical role of mutated (activated) *ras* genes in carcinogenesis was confirmed in experimental cancer systems. Activated c-Ha-*ras* genes were reproducibly detected in skin papillomas and carcinomas of mice treated with dimethylbenzanthracene (Balmain et al. 1984). Mouse mammary carcinomas induced by nitrosomethylurea consistently harbored Ha-*ras* mutations (Sukumar et al. 1983; Zarbl et al. 1985). Exposure to the same carcinogen or gamma-radiation of mice resulted in the formation of thmyic lymphomas carrrying N-*ras* mutations (Guerrero et al. 1984a,b). A comprehensive list of *ras* mutations associated with carcinogen exposure has been reported by Barbacid (1987). More recently, transgene technology has been used to target the expression of activated *ras* genes to defined tissues. N-*ras* or Ha-*ras* oncogenes were cloned under the control of a chimeric immunoglobulin (IgH) enhancer/SV40 promoter, of the mouse mammary tumor virus long terminal repeat (MMTV-LTR), whey acidic protein (WAP) promoter, elastase promoter, and growth hormone releasing factor (GHRF) promoter. Transgenic mice carrying these gene constructs gave rise to neoplasia. The tumors included T-cell lymphomas (N-*ras* gene controlled by IgH-SV40 enhancer/promoter), lung adenocarcinomas (IgH-SV40/Ha-*ras*), mammary carcinomas (MMTV-LTR/Ha-*ras*), and neonatal acinar cell hyperplasia (elastase promoter/Ha-*ras*). Rare mammary tumors were detected in mice carrying WAP/Ha-*ras* transgenes, and no phenotypic changes could be observed in mice with the GHRF/Ha-*ras* transgene (for review see Hanahan 1988; Pattengale et al. 1989; Cardiff et al. 1991).

Ras proteins bind guanine nucleotides (GDP and GTP). The bound GTP is hydrolyzed to GDP and inorganic phosphate. These biochemical properties of $p21^{ras}$ are essential for its biological function. When anti-*ras* antibodies were microinjected into *ras*-transformed NIH/3T3 cells, guanine nucleotide binding was inhibited, and neoplastic transformation was abolished. Mutated Ha-*ras* genes that do no longer bind guanine nucleotides were unable to neoplastically transform NIH/3T3 cells. Transforming alleles of *ras* genes carrying point mutations exhibited a tenfold decrease of GTPase activity together with accumulation of the GTP-bound form of the protein. It was concluded that Ras receives a signal from a molecule located upstream in the signaling pathway and that the activated protein transmits this stimulus to a downstream effector most probably via interaction by its effector region (for review see Barbacid 1987; Grand and Owen 1991; Satoh

et al. 1992). The levels of GDP and GTP bound to Ras were determined in intact normal cells. There are various extracellular stimuli able to activate wild-type Ras by enhancing the proportion of GTP-bound protein in different types of cells. These include not only the neoplastic transformation of fibroblast cells by receptor-type or non receptor-type oncogenes (Gibbs et al. 1990; Satoh et al. 1990), but also the stimulation of normal cells by growth factors such as PDGF, EGF, and insulin (Mulcahy et al. 1985; Cai et al. 1990; Medema et al. 1991). Stimulation of T cell, B cell, and mast cell lines by different interleukins and colony-stimulating factors lead to accumulation of Ras-GTP (Satoh et al. 1991; Duronio et al. 1992; Graves et al. 1992; Heidaran et al. 1992). In erythroleukemic cell lines, Ras was activated by erythropoeitin (Torti et al. 1992). Activation of T lymphocytes appeared to be mediated via Ras-GTP (Downward et al. 1990; Graves et al. 1991). However, Ras-GTP is not only involved in signaling events that trigger cellular proliferation. Ras proteins may function as transducers of neuronal differentiation signals in rat PC12 pheochromocytoma cells (Noda et al. 1985; Bar-Sagi and Feramisco 1985), of adipocytic conversion in murine 3T3-L1 fibroblasts (Benito et al. 1991), of steroidogenic differentiation of ovarian surface epithelial cells (Pan et al. 1992), and of mesoderm induction in *Xenopus* embryogenesis (Whitman and Melton 1992). In addition, growth inhibition of epithelial cells by transforming growth factor β is associated with GTP binding to Ras and thus dependent on Ras signal transduction (Mulder and Morris 1992). Expression of v-Ha-*ras* lead to growth arrest in Schwann cells that express a temperature-sensitive mutant of SV40 large T antigen at the restrictive temperature (Ridley et al. 1988). Ras proteins are components of a cascade of signaling events rather than of an unidirectional signal transduction pathway. A number of molecules have been identified which modulate Ras activity by interfering with the state of bound guanine nucleotides (for review see Satoh et al. 1992). The signaling events between a receptor tyrosine kinase located at the cell surface and Ras have very recently been elucidated. Several groups have described physical interactions between the internal domain of ligand-activated kinase receptors and GrB2 protein, which binds to mSos1, a Ras nucleotide-exchange protein. Receptor-activated Sos facilitates GDP to GTP exchange on Ras (for review see McCormick 1993). Active GTP-Ras feeds the signal into a cascade of serine-threonine kinases which send it further to the nucleus (for review see Roberts 1992).

2 Limited Contribution of Mutant *ras* Genes to Malignant Transformation

The expression of transfected *ras* oncogenes in preneoplastic mouse NIH/3T3 cells resulted in morphological transformation, anchorage-independent growth, and tumorigenicity in nude mice. The intrinsic GTPase activity of Ras is greatly diminished upon mutation. Therefore, the transforming activity of the mutated protein is explained by the preferential maintenance of the active, GTP-bound state. Increasing the proportion of Ras-GTP by overexpression of the normal *ras* allele may result in the same phenotype (for review see Barbacid 1987). Since NIH/3T3 cells are immortal and exhibit an aneuploid phenotype, it is conceivable that genetic alterations facilitating the transforming activity of oncogenes have already occurred. The genome of normal diploid embryo fibroblasts provides a more efficient barrier against the deleterious effects of *ras* oncogenes. Neoplastic transformation of rat embryo fibroblasts was not achieved by expression of an activated *ras* gene alone, but required the simultaneous expression of a cooperating oncogene such as *myc* or adenovirus E1A (Land et al. 1983; Ruley 1983). Oncogene cooperation was also demonstrated in vivo (Paterson et al. 1989). Since transformed cell lines obtained after transfection of oncogenes or tumors emerging in oncotransgene-carrying mice were usually not analyzed by karyotyping, important gross genetic alterations might have escaped attention. Tumor formation by transformed Syrian hamster embryo fibroblast required the expression of cooperating *ras* and *myc* oncogenes and the loss of a putative tumor suppressor gene as indicated by consistent chromosomal deletions (Oshimura et al. 1985) or by the loss of regulatory signals from neighboring cells mediated by gap-junctional communication (Land et al. 1986). Growth factor-independent proliferation of murine hematopoietic cells was associated with a great variability in their karyotypes (Vogt et al. 1986). Neoplastic transformation of rat embryo fibroblasts mediated by overexpression of mutated *ras* alone as described in one report (Spandidos and Wilkie 1984) can possibly be explained by similar undetected genetic alterations. Most importantly, although all cells in a given tissue to which expression of oncotransgenes has been targeted provide the necessary transcriptional machinery for their activity, it is only a rare fraction of cells that give rise to malignant growth. Consequently, tumors obtained in mice carrying oncogenes as transgenes were mono- or oligoclonal, suggesting that additional changes had occurred (for review see Pattengale et al. 1989; Cardiff et al. 1991). Similarly, tumors derived after infection of midgestation mouse embryos with recombinant retroviruses carrying *ras* and *myc* oncogenes were of monoclonal origin. Thus, additional alterations

were necessary for the realization of the fully malignant phenotype (Compere et al. 1989).

Molecular genetic studies in human tumors support the notion that the malignant state is characterized by a set of genetic alterations including oncogene activation and loss of suppressor gene function. For example, Ki-*ras* genes are not only frequently mutated (for review see Bos 1989), but also play an important functional role in colorectal tumors. Disruption of this oncogene in two human colon carcinoma cell lines by homologous recombination resulted in morphological alterations, loss of anchorage-independent proliferation, and reduction in tumor formation in nude mice (Shirasawa et al. 1993). The mutational activation of a *ras* oncogene did not induce the transformed phenotype in a dominant manner, but rather predisposed a nontumorigenic immortalized cell line to transformation by independent events. This was shown by the replacement in Rat-1 fibroblasts of one of the normal Ha-*ras* alleles by a mutated copy via homologous recombination (Finney and Bishop 1993). The transition from normal colonic epithelium to a malignant tumor capable of shedding metastatic cells appears to involve the functional inactivation of several transformation suppressor genes (Fearon and Vogelstein 1990). Tumor suppressor genes of relevance for both hereditary and sporadic colorectal tumors include the *AFP* gene (Kinzler et al. 1991), *p53* (Baker et al. 1989), and the *DCC* gene (Fearon et al. 1990). The number of genetic lesions, involved in colorectal carinogenesis is still increasing (Peltomäki et al. 1993; Aaltonen et al. 1993). Other human tumors may have sustained a number of mutations in some of those genes as well as in genes located on different chromosomes. The *p53* gene acts as a suppressor of proliferation and neoplastic transformation in human tumor cell lines (Baker et al. 1990; Goyette et al. 1992). Circumstantial evidence suggests that the *DCC* gene which encodes a cellular adhesion protein can also restrain malignant proliferation (Narayanan et al. 1992).

The deletions and/or mutations in tumor suppressor genes found in transformed cells with activated *ras* oncogenes suggest that the constitutively activated signal transduction pathway does not by itself mediate uncontrolled proliferation. The changes occurring in *ras*-expressing cells comprise irreversible mutational events. Recently it was demonstrated that *ras* oncogene expression increased both the frequency of chromosomal aberrations and of spontaneous mutations in the hypoxanthine phosphoribosyl-transferase gene (Baron et al. 1992). The *ras*-mediated transformation is associated with the modulation of gene expression. These alterations include both enhanced expression of cellular genes as well as downregulation of gene activity (for review see Klemenz and Aoyama 1993).

3 Suppression of *ras*-Mediated Neoplastic Transformation

3.1 Cell Fusion Studies

Several lines of evidence have suggested that the neoplastic phenotype of cells expressing *ras* oncogenes can be reversed. Somatic cell hybridization of transformed and normal cells had suggested that normal cells contain transformation-suppressing activity. The suppression of tumorigenicity and of anchorage-independent proliferation appeared to have a genetic basis. Whereas the suppressed hybrids often contained the full chromosomal complement of both parental genomes, reemergence of transformed phenotypes in cell segregants was associated with loss of chromosomes derived from the normal parental genome (for review see Stanbridge 1991). Several groups have reported on the isolation of suppressed somatic cell hybrids between *ras* oncogene-expressing cell lines and normal cells. A nontransformed Chinese hamster embryo fibroblast cell line (CHEF) was fused with a hamster transfectant line carrying the human mutant c-Ha-*ras* gene (Craig and Sager 1985). Selected hybrids exhibited a fibroblastic morphology, had a reduced capacity for anchorage-independent growth and decreased tumor-forming ability in nude mice. Suppressed hybrid clones contained the mutant *ras* gene and expressed its p21 protein product at levels comparable to the transformed parental cells. Somatic cell hybrids of Rat-1 cells transformed with the activated c-Ha-*ras* gene from human EJ bladder carcinoma cells and diploid embryonic rat fibroblasts expressed one-tenth to one-third of the mutant $p21^{ras}$ product as compared to parental cells. The hybrid cells showed the suppressed phenotype. It was concluded that the transforming activity of the activated Ha-*ras* gene can be suppressed at the posttranslational level by the presence of the genome of normal fibroblasts (Griegel et al. 1986). The v-Ha-*ras* oncogene was introduced into a mouse mastocytoma cell line which acquired the capacity to produce interleukin-3 (IL-3) and to initiate tumor growth. When this cell line was fused with its IL-3-dependent, nontransformed parental cell line, hybrid cell lines dependent on IL-3 for proliferation in vitro were isolated. The endogenous IL-3 production was down-regulated in these hybrids. Although the $p21^{ras}$ protein was expressed at high levels, the tumor-forming potential of hybrid cells was reduced, as indicated a by fivefold prolonged latency period (Diamantis et al. 1989). The authors concluded that a tumor suppressor present in mouse mastocytes acts as a negative regulator of IL-3 expression and is responsible for the loss of autonomous growth.

The suppression of *ras*-transformation is not restricted to rodent cell hybrids. When human EJ bladder carcinoma cells, harboring a mutant Ha-*ras* allele, were fused with a normal human fibroblast line, hybrids were

isolated that behaved as transformed cells in culture but did not form tumors in nude mice (Geiser et al. 1986). The hybrid cells maintained the suppressed phenotype, even if the level of Ha-*ras* expression was elevated by transfection of additional copies of the oncogene. Results of cell fusion experiments involving different human cell lines expressing either Ha-*ras*, Ki-*ras*, or N-*ras* oncogenes suggested that suppression of tumorigenicity can occur even in the presence of two transforming genes of the *ras* family (Geiser et al. 1989). Human uroepithelial cells immortalized by SV 40 were transfected with the *ras* oncogene derived from EJ bladder carcinoma cells. Tumorigenic transformation of these epithelial cells occurred as a rather rare event. Cell fusion of a tumorigenic transformant, and a nontumorigenic transfectant clone indicated the suppression of tumorigenicity in spite of mutant $p21^{ras}$ expression (Pratt et al. 1992). The ability to block neoplastic transformation mediated by N-*ras* of PA-1 human teratocarcinoma cells was dependent on the duration of cell culture (Krizman et al. 1990). While early-passage PA-1 cells were resistant toward the oncogene, late-passage cells could be transformed with high efficiency. Fusion of low- and high-passage cells showed that the resistance toward transformation is a dominant trait. A *ras*-resistant derivative of PA-1 teratocarcinoma cells cannot be stimulated to grow without anchorage by growth factors added to the medium (Chiao et al. 1991).

The results from cell fusion studies using *ras*-expressing cells suggest that the transformed properties in vitro and in vivo are controlled by unknown tumor suppressor genes. The ability to metastasize may be suppressed as well since the metastatic potential of v-Ha-*ras* expressing rat mammary cells was abolished upon fusion with the nonmetastatic dimethylbenz[a]anthracene-induced parental line. The hybrid clones maintained their ability to form primary tumors (Ichikawa et al. 1992). All hybrid clones continued to express the *ras* oncogene.

To identify chromosomes which carry putative tumor suppressor genes, Yamada et al. (1990) introduced microcells harboring either human chromosomes 1, 11 or 12 tagged with pSV2neo DNA into Kirsten sarcoma-virus transformed NIH/3T3 cells. The growth rate, colony-forming ability in semisolid agar medium and tumorigenicity in nude mice was reduced in Ki-*ras* transformed cells carrying chromosome 1, but not in those cells into which chromosome 11 or 12 was inserted. Recently, growth suppression was shown in human RD rhabdomyosarcoma cells which harbor an activated N-*ras* gene (Hall et al. 1983). A modification of the microcell fusion technique was used that permits transfer of subchromosomal fragments rather than individual chromosomes (Koi et al. 1993). Microcells were prepared from mouse A9 cells harboring human t(11;X) chromosomes as the only exogenous chromosomes tagged with multiple copies of the *neo* gene. The

microcells were gamma-irradiated to generate subchromosomal transferable fragments (STF). Microcell-mediated fusion of irradiated chromosomal fragments into A9 cells allowed the propagation and subsequent transfer of small defined parts of this human chromosome. With the help of chromosome-specific DNA markers the precise origin of human chromosomal fragments transferred into A9 cells was determined. When ten different STF specific for chromosome band 11p15 were introduced into RD rhabdomyosarcoma cells, eight microcell fusions gave rise to colonies consisting of flat, enlarged, and elongated cells. These colonies ceased to proliferate 2–4 weeks after microcell fusion. By comparison with STF that did not transmit antiproliferative activity, it was concluded that the suppressive activity is associated with band 11p15.5.

3.2 *ras*-Resistant Cell Lines

Established rodent cell lines have been described that cannot be stably transformed by overexpressed *ras* oncogenes. Introduction of activated *ras* failed to transform REF52 cells. Tumorigenic transformation required collaborating adenovirus E1A or simian virus large tumor (T) antigen oncogenes (Franza et al. 1986; Hirakawa and Ruley 1988). Thus, immortalized REF52 cells resemble normal rat embryo fibroblasts. The resistance toward *ras* transformation indicates that immortalization per se is not a sufficient prerequisite for tumorigenic conversion by this oncogene. In the presence of E1A expression, higher levels of $p21^{ras}$ were accumulated in REF52 cells (Franza et al. 1986). Elevating the level of $p21^{ras}$ in the absence of E1A resulted in cell growth arrest and morphological crisis. A derivative of mouse NIH/3T3 cells, designated EK-3, exhibits a similar resistance toward transformation by a *ras* oncogene alone (Katz and Carter 1986; Katz and Samid 1989). Elevated levels of Ha-*ras* mRNA were insufficient to induce morphological transformation and anchorage-independent proliferation in this cell line. In addition, a number of gene transfer experiments using *ras* oncogenes alone or in combination with a collaborating oncogene have been reported that failed to transform normal human recipient cells. The efficient expression of oncogene products was demonstrated in these cells (Stevenson and Volsky 1986; Sager et al. 1986). The finite life-span of human recipient cells appeared to limit the expression of malignancy, since *ras* oncogenes were able to transform immortalized, nontumorigenic human cells (Hurlin 1989; Seremetis et al. 1989; Wilson et al. 1990; Fry et al. 1990).

4 Phenotypic Revertants Derived from *ras*-Transformed Cells

4.1 Oncogene, Suppressor, and Effector Mutants

Morphologically altered (flat) revertants were obtained from cell lines transformed by the viral *ras* oncogene. Flat revertants were isolated from populations of cells transformed by Kirsten murine sarcoma virus by cell "suicide" techniques. The principle of these methods is that cells were cultured under conditions which favor the proliferation of transformed cells but result in growth arrest of revertant cells. Those cells were protected because of their mitotic inactivity. Transformed cells were mutagenized prior to the selection procedure to increase the frequency of phenotypic reversion. The selective culture conditions included treatment with 5-fluorodeoxyuridine at high density, in methylcellulose (Ozanne and Vogel 1974) or in serum-poor medium (Vogel and Pollack 1974), with concanavalin A (Ozanne and Vogel 1974), with iododeoxyuridine in methylcellulose (Greenberger and Aaronson 1974), and with high temperature (Cho et al. 1976). Revertant cell lines were also isolated from clonal lines of virus-transformed cells at very low frequency by selection with bromodeoxyuridine (Stephenson et al. 1973; Morris et al. 1980; Norton et al. 1984). The loss of transformed characteristics resulted from mutations affecting the expression of the functional viral oncogene (Ozanne and Vogel 1974; Greenberger and Aaronson 1974; Cho et al. 1976; Vogel and Pollack 1974). Other revertant cell lines had sustained mutations in cellular effector genes necessary for the expression of the transformed phenotype (Stephensen et al. 1973; Morris et al. 1980; Norton et al. 1984). The early work on phenotypic revertants has been reviewed comprehensively by Bassin and Noda (1987). The expression of neoplastic transformation may also be modulated by changes in the dosage of the oncogene. Revertants derived from human HT1080 fibrosarcoma cells after mutagenesis and cell "suicide" selection showed an increase in chromosomal ploidy, while the one mutated allele of activated N-*ras* persisted. The level of the mutant $p21^{N\text{-}ras}$ product was therefore decreased in the revertants relative to the parental cell line. Introduction of another copy of the transforming allele resulted in retransformation of the revertants (Paterson et al. 1987).

Cell lines that continue to express the oncogene despite the loss of transformed properties were of particular interest for the study of the mechanisms of oncogene resistance. DT, a clone of NIH/3T3 cells containing two copies of the Ki-MuSV genome, was used for the isolation of flat revertants (Noda et al. 1983). The number of flat colonies resulting from mutations in the v-*ras* gene was minimized by the presence of two integrated copies of the virus in this cell line. When *ras*-transformed DT cells were treated with the

mutagen 5'-azacytidine and selected in medium depleted of K^+ and supplemented with ouabain, the *ras*-transformed cells showed a significantly lower efficiency of cloning in the presence of the drug. In contrast, normal NIH/3T3 and other cell lines not expressing activated *ras* genes are relatively insensitive toward ouabain-treatment (Benade et al. 1986; Talbot et al. 1988; Wang et al. 1988). Noda et al. (1983) chose two ouabain-resistant flat colonies for further analysis. These two revertant cell lines had lost several properties associated with *ras* transformation. These properties included a short doubling time, the ability to form colonies in semisolid agar medium and to initiate tumor growth in nude mice. In spite of the expression of the nontransformed phenotype, the cells continued to express $p21^{ras}$ at an unaltered level. Transforming virus particles could be rescued from revertant cell lines. The authors concluded that the two revertant cell lines possess alterations in their cellular genomes that allow them to block the Ki-MuSV-induced transformation. The block occurred at some point distal to the initial interaction of p21 with its target molecules. The revertant cell lines were fused with cell lines transformed by Ha-MuSV, BALB-MuSV, v-*mos*, v-*fes*, v-*fms*, v-*sis*, v-*src* oncogenes, or polyomavirus. The resulting somatic cell hybrids were analyzed for their ability to proliferate in semisolid agar medium. Neoplastic transformation triggered by *ras*, *fes*, and *src* oncogenes was suppressed in cellular hybrids, whereas *mos*-, *fms*-, and *sis*- and polyomavirus-transformed cells were unaffected (Table 1).

Kuzumaki et al. (1989) isolated a flat revertant clone, designated R1, from NIH/3T3 cells transformed by an activated human Ha-*ras* gene after mutagenesis. R1 cells were resistant to the transforming activity of Kirsten sarcoma virus, the cellular mutant Ha-*ras* gene, v-*src*, v-*mos* oncogenes, SV40 large T antigen, or polyoma middle T antigen. To find out whether R1 cells had sustained mutations in cellular genes that interfered with the expression of the neoplastic phenotype, the authors performed cell fusion of R1 and NIH/3T3 cells. Somatic cell hybrids expressed the nontransformed phenotype suggesting the activation of transformation suppressor genes in R1.

Two new revertant clones have been isolated from the *ras*-transformed cell line DT using *cis*-4-hydroxy-L-proline (CHP) as a selective agent (Yanagihara et al. 1990). CHP is able to interfere with the synthesis of proteins that are rich in proline and hydroxyproline such as collagen. Since normal collagen production is necessary for cell growth, CHP acts as a growth inhibitor. Rodent cells transformed by different oncogenes, RNA and DNA tumor viruses or by the carcinogen nitrosomethylurea are more susceptible to the antiproliferative properties of CHP than are their untransformed precursor cells (Ciardiello et al. 1988). Although the two CHP-resistant revertants continued to express the v-Ki-*ras* oncogene and contain rescuable virus, they were anchorage-dependent and only weakly tumo-

Table 1. Phenotypic revertants: pattern of resistance toward oncogene-mediated transformation

Parental cell line	Oncogene	Mode of revertant selection	*ras*	*src*	*fes*	*fgr*	*fms*	*abl*	*mos*	*raf*	*erb-B2*	*sis*	*fos*	Pol mT
NIH/3T3	v-K-*ras*	AC, ouabain	+	+	+		–		–			–		–
NIH/3T3	v-K-*ras*	EMS, CHP	+	–	–	–	–		+	+		–	+	
NIH/3T3	v-K-*ras*	EMS, ouabain	+	+	+	+	–		–	–		–	–	
NIH/3T3	c-H-*ras* mutant	EMS, AzaG	+	+					+					+[a]
NIH/3T3	c-H-*ras* mutant	BrdU + irradiation	+					(+)			–	–		
NIH/3T3	LTR-c-H-*ras*	Interferon α/β	+		+			+						
NIH/3T3	c-H-*ras* mutant	Interferon γ	+	(-)	(-)			(-)	+					[b]
NIH/3T3	c-H-*ras* mutant	Azatyrosine	+	+						+				[c]
BALB/c	v-*mos*	Interferon	(-)				+		+			+	+	
Rat-1	v-*fos*	EMS, Rhodamine	+					+	+				+	–[d]
208F	v-*fos*	Adhesion	+										+	
Rat6	c-H-*ras* mutant inducible	Resistance to TPA/$ZnSO_4$	+	+					+				(+)	
F2408	v-K-*ras*, ts	UV Irradiation	+	+					+		+		+	–[e]
CCL 64	v-*fes*	MNNG rhodamine	+		+		+							

+, Cells resistant to transformation by indicated oncogene; –, cells sensitive to transformation; (+), heterogeneous response to oncogene expression; (–), revertants partially retransformed. AC, 5'-Azacytidine; EMS, ethane methosulfonate; CHP, *cis*-4-hydroxy-L-proline; AzaG, 8-azaguanine; BrdU, bromodeoxyuridine; TPA, 12-O-tetradecanoylphorbol 13-acetate; ts, temperature-sensitive mutant;
MNNG, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine.

[a] Cells resistant to SV40 large T.

[b] Cells partially transformed by v-*myc*.

[c] Cells sensitive toward *hst*/*ret*.

[d] Cells sensitive to *trk*.

[e] Cells sensitive to *trk*, human papilloma virus, adenovirus type 12.

References see text.

rigenic. The oncogene resistance pattern of these CHP-derived revertants was compared with those from revertants derived from ouabain selection (Noda et al. 1983). Oncogenes were divided into four groups depending on their transforming activity when expressed in revertants or on their inability to confer the neoplastic phenotype. (Yanagihara et al. 1990). Oncogenes of the *ras* family form the first group in that they are suppressed by both ouabain-selected and CHP-selected revertants. Oncogenes with tyrosine kinase activity (*fes*, *src*, *fgr*) were suppressed by ouabain-selected revertants, but were able to retransform CHP-selected revertants. Group three includes oncogenes whose transforming activity is inhibited in CHP revertants, but not in ouabain revertants. Members of this group are oncogenes encoding serine-threonine kinases (*mos*, *raf*) and the nuclear oncogene *fos*. The oncogenes *sis* and *fms* were able to retransform both ouabain and CHP revertants and form group four. These results imply that many oncogene products exert their transforming activity via identical biochemical pathways. Thus, the number of different transformation pathways may be limited.

Two serum- and anchorage-dependent revertant cell lines were isolated from Ha-*ras* transfected NIH/3T3 cells by incubation in serum-free medium with bromodeoxyuridine. The revertants retained their nontransformed phenotype even if the level of mutated $p21^{ras}$ was elevated by the introduction of additional copies of the oncogene (Yamada et a. 1990). Somatic cell hybrids generated by fusion of each of the revertant cell lines with nontransformed NIH/3T3 cells expressed the transformed phenotype. Fusion of the two revertant cell lines with each other resulted in a hybrid culture showing decreased anchorage requirements but only a slightly decreased serum dependence. The authors concluded that the revertants are recessive and have sustained mutations in effector genes necessary for the expression of the neoplastic phenotype. The significant reduction of anchorage dependence in the revertant x revertant fusion suggested that there may be two different complementation groups. Retransformation experiments with four types of oncogenes showed that the revertants and NIH/3T3 cells had an equal sensitivity toward c-*sis*, c-*neu*, and polyoma middle T oncogenes as indicated by colony growth in semisolid agar medium. In contrast, one revertant clone was relatively insensitive toward transformation by the v-*abl* oncogene.

The cell line B812, a derivative of Fisher rat fibroblasts, is a temperature-dependent cell mutant that shows a diminished frequency of foci after infection with different retroviruses (Inoue et al. 1983). For example, upon infection with Kirsten murine sarcoma virus the number of foci decreased more than 25-fold at the nonpermissive temperature of 39°C compared to the parental fibroblast line. Despite this phenotypic difference, the cells expressed nearly the same level of the *ras* transcript and $p21^{ras}$ protein at the permissive and nonpermissive temperature. The ability to restrict focus

formation was completely suppressed, when B812 cells were fused with wild-type parental cells. The temperature dependence was found after transformation with the oncogenes *mos*, *fos*, *src*, and *erbB2* but not after transformation mediated by polyomavirus middle-T antigen, adenovirus type 12, and human papillomavirus 16. These results suggested that the temperature-dependent transformation of B812 cells resulted from a mutation in a cellular factor(s) that is a common component of the transformation pathway used by *ras*, *mos*, *fos*, *src*, and *erbB2* oncogenes (Kizaka and Hakura 1989).

Revertants were obtained from transformed cells that expressed oncogenes other than *ras*. These cells exhibited resistance toward retransformation by *ras*, suggesting that some oncogenes exert their transforming activity via identical biochemical pathways. Morphological revertants of FBJ murine sarcoma virus-transformed Rat-1 cells were isolated after mutagenesis using a selection procedure based on the prolonged retention of the dye rhodamin-123 in mitochondria of transformed versus normal cells (Zarbl et al. 1987). The revertants continued to express the v-*fos* oncogene but exhibited features of nontransformed cells including loss of anchorage-independent growth and tumorigenicity. The revertants were resistant to retransformation by v-*gag-fos-fox*, v-Ha-*ras*, v-*abl*, and v-*mos* oncogenes but could be retransformed by the *trk* oncogene and polyoma virus middle T antigen. Somatic cell hybridization studies showed that the revertant phenotype was recessive, suggesting that the revertant cells have sustained mutations in cellular genes (or a single cellular gene) essential for neoplastic transformation. Following mutagenesis of rat fibroblasts transformed by Finkel-Biskis (FBR) murine sarcoma virus, revertants were isolated on the basis of their adherence to plastic culture dishes in the absence of divalent cations. These revertants were flat, contact-inhibited when grown to confluence, anchorage-dependent, and nontumorigenic in nude mice. Somatic cell hybrids of these revertants and the v-*fos*-transformed parental cells were nontransformed, suggesting the existence of a transformation-suppressing gene. These revertants were resistant to transformation by v-*fos*, c-*jun*, and activated c-Ha-*ras* genes (Wisdom and Verma 1990).

A revertant cell line of mink cells transformed by the Gardner-Arnstein strain of feline sarcoma virus showed a decrease in its proliferative capacity and saturation density, and a complete loss of its ability to form colonies in semisolid agar medium. The cell line was, however, tumorigenic in nude mice. Feline sarcoma provirus could be rescued from this cell line, and high levels of the v-*fes* oncogene were expressed. The revertant line was refractory toward transformation by retroviruses carrying v-*fes*, v-*fms*, and v-*ras* oncogenes. The transformation block was recessive, as fusion of the revertant line with the parental line yielded transformed hybrid cells. These results suggested that a defect in or limiting quantity of a gene product which

functions downstream of the kinase and *ras* proteins abolished transformation in vitro (Haynes and Downing 1988).

4.2 Revertants Obtained After Treatment with Cytokines, TPA, Inducers of Differentiation, and Antibiotics

Revertants which maintained a nontransformed phenotype were obtained from NIH/3T3 cells transformed by a LTR-activated Ha-*ras* proto-oncogene by prolonged treatment with IFN-α/β (Samid et al. 1987). The nontransformed phenotype was maintained even after the cytokine treatment was discontinued (persistent reversion). Moreover, the cells continued to express large amounts of $p21^{ras}$ and were resistant to transformation by transforming retroviruses harboring v-Ha-*ras*, v-Ki-*ras*, v-*abl*, or v-*fes* oncogenes. Retransformation was achieved by treating persistent IFN revertants with cytidine analogs. The authors suggested that DNA methylation is involved in IFN-triggered reversion. A revertant cell line was isolated from Moloney sarcoma virus transformed BALB/c cells after long-term IFN treatment (Gerfaux et al. 1990). The cells were not retransformable with viruses carrying v-*fes*, v-*fms*, and v-*fos* oncogenes. Infection with Kirsten sarcoma virus (v-Ki-*ras*) did not alter the flat morphology of revertant cells in vitro, but resulted in tumorigenicity. Persistent reversion was also achieved by treatment with IFN-γ of NIH/3T3 fibroblasts transformed by a mutant human Ha-*ras* oncogene (Seliger et al. 1991). Long-term treatment with the cytokine was not required. Rather, the population of cytokine-treated cells was subjected to the ouabain selection procedure described by Noda et al. (1983). The revertants resisted the transforming activity of v-Ha-*ras* and v-*mos* oncogenes but were partially retransformed by treatment with 5-azacytidine or by infection with recombinant retroviruses carrying the v-*abl*, v-*fes*, v-*myc*, or v-*src* oncogenes.

Krauss et al. (1992) took advantage of a rat cell line that stably overproduces protein kinase $C\beta_1$ and harbors an activated c-Ha-*ras* oncogene controlled by an inducible metallothionein I promoter (MT I). Treatment of cells with TPA and $ZnSO_4$ led to cell death. However, surviving colonies arose in cell cultures either spontaneously or after induction by the mutagen ethyl methane sulfonate. Two variant cell lines, resistant to the cytocidal effects of TPA and $ZnSO_4$, showed a flat phenotype and did not grow in soft agar. The revertants continued to express $PKC\beta_1$ and retained an intact *ras* oncogene. These revertants were resistant to neoplastic transformation by v-*src* and v-*raf*. One of the two cell lines was refractory toward v-*fos*-induced transformation as well. When the two revertant cell lines were treated with TPA or infected with a retrovirus harboring an LTR-controlled Ha-*ras* oncogene,

they regained a morphologically transformed phenotype but retained a strict anchorage dependence. Thus, *ras*- and PKC-mediated events leading to morphological transformation can be dissociated from those events involved in anchorage-independent growth. Moreover, the revertants failed to express the inducible exogenous *ras* oncogene as well as endogenous MT I and MT II genes on stimulation by the inducer. The authors proposed the existence of a *trans*-acting factor that represses the activity of genes controlling anchorage-independent growth. These unknown genes might have MT-like regulatory elements (Krauss et al. 1992).

Following treatment with the maturational agent sodium butyrate, the poorly differentiated human colon carcinoma cell line MIP-101 reverted to a more normal phenotype in that its population doubling time increased, the capacity to form colonies in semisolid agar medium was eliminated, and tumorigenicity was reduced (Niles et al. 1988). MIP-101 cells harbor an activated N-*ras* oncogene. The expression of the reverted phenotype was not correlated with decreased transcription of the oncogene (Stoddart et al. 1989). However, there was a marked reduction in the transforming activity of the genomic DNA from butyrate-treated MIP-101 cells. The molecular basis of this loss of transforming potential is not understood; however, the phenomenon appeared to be specific to MIP-101 cells. When a NIH/3T3 transformant harboring the human N-*ras* gene was treated with butyrate, similar phenotypic effects were observed. In contrast to MIP-101 cells, the transforming activity of genomic DNA from this cell line was unimpaired after butyrate-treatment. Flat revertants of v-*ras*-transformed rat kidney cells (KNRK) were generated to high efficiencies with sodium butyrate. The revertants expressed elevated levels of $p21^{ras}$ (Ryan and Higgins 1989; Higgins and Ryan 1989).

The antibiotic azatyrosine selectively inhibited the growth of NIH/3T3 cells transformed by the activated human c-Ha-*ras* gene, while the growth of untransformed cells was unaffected (Shindo-Okada et al. 1989). More than 85% of azatyrosine-treated *ras*-transformed cells converted permanently to revertant cells that exhibited a flat morphology, contact inhibition, anchorage dependence, and a reduction of tumorigenicity in nude mice. The expression of the *ras* oncogene was unaffected. A similar reversion was caused by azatyrosine treatment of NIH/3T3 cells transformed by activated c-Ki-*ras*, N-*ras* and c-*raf* genes and of human pancreatic adenocarcinoma cells (PSN-1) harboring amplified Ki-*ras* and *myc* genes. In contrast, cells transformed by *hst* and *ret* oncogenes were refractory toward azatyrosine treatment. A summary of the pattern of resistance and sensitivity toward oncogene-mediated transformation observed in phenotypic revertants is shown in Table 1. The mode of azatyrosine action is not understood. It is very likely that azatyrosine affects the expression of effector genes necessary for signal

transduction triggered by *ras* and other oncoproteins (Shindo-Okada 1989). Azatyrosine-induced revertant cell lines were also isolated from v-Ha-*ras* transformed human mammary epithelial cells (Kyprianou and Taylor-Papadimitriou 1992). Those cells were phenotypically nontransformed and nontumorigenic in nude mice, even though they contained prereversion levels of *ras* mRNA and p21 protein. Similarly, the expression of $p21^{ras}$ was not affected in revertants of *ras/myc* transformed BALB/c mouse embryo cells cultured in serum-free medium (Nomura et al. 1992).

Nalidixic acid (NaI) is an antibacterial agent that inhibits bacterial DNA gyrase, eukaryotic topoisomerase II, and viral replication in cultured cells. NaI reversibly suppressed growth in soft agar and reduced saturation density of BALB/3T3 cells transformed by methylcholantrene or of NIH/3T3 cells transformed by an activated c-Ha-*ras* oncogene (Kaneko and Horikoshi 1989). The amount of $p21^{ras}$ detected in NaI-treated cells was indistinguishible from control transformed cells. Two related compounds, oxolinic acid and pipemidic acid, were less effective than NaI in suppressing transformed cells. The authors speculate that these compounds indirectly modulate gene expression by interfering with topoisomerase activity.

Rat kidney cells infected with ts371 Kirsten murine sarcoma virus expressed the normal phenotype at the nonpermissive temperature (39.5°C) and the transformed phenotype at the permissive temperature (34°C; Itoh et al. 1989). Normal cells were characterized by expression of an unstable and less palmitylated $p21^{Ki\text{-}ras}$ and low GTP levels. When cells were treated with oxanosine, a guanine analogue antibiotic, at the permissive temperature, the transformed phenotype was blocked. GTP levels and $p21^{Ki\text{-}ras}$ stability resembled that of cells grown at the nonpermissive temperature.

A transformation-suppressed cell line R35, which accumulated a 23-kDa precursor of $p21^{ras}$, was isolated from *ras*-transformed NIH/3T3 cells. The revertant cell line was retransformed upon fusion with nontransformed Rat-2 cells. The defect that inhibited the expression of neoplastic transformation in R35 cells was due to a slower rate of palmitylation and polyisoprenylation of *ras* proteins (Huang and Axelrod 1991). Transformation by *ras* oncogenes can also be blocked at the posttranslational level by interfering with farnesylation that is necessary for membrane attachment of $p21^{ras}$ (for review see Schafer et al. 1989; Schafer and Rine 1992; Marshall 1993). Farnesyl synthesis and $p21^{ras}$ farnesylation can be blocked by lovastatin, an inhibitor of hydroxymethylglutaryl coenzyme A reductase. Lovastatin was shown to reverse abnormal morphology and loss of anchorage dependence as well as to reduce tumorigenicity in v-Ha-*ras* transformed rat liver epithelial cells (WB-*ras*; Ruch et al. 1993). In addition, lovastatin enhanced gap-junctional intercellular communication (GJIC) in WB-*ras* cells, suggesting that reduced GJIC plays a role in the expression of the transformed state (for review see Yamasaki 1991).

4.3 Reversion Induced by Inhibition of *ras* Expression

Benzamide (BA) is a NAD^+ site inhibitor of poly(ADP-ribose) polymerase which modifies numerous proteins by poly(ADP) ribosylation. This post-translational protein modification is involved in DNA repair, DNA replication, sister chromatid exchange, and cell differentiation. Probably, perturbed areas of chromatin generated around the regions of integration of exogenous genes are removed, when poly(ADP-ribose) polymerase inhibitors are active. BA induced a flat morphological phenotype of NIH/3T3 cells transformed by insertion of activated Ha-*ras*, Ki-*ras*, or N-*ras* genes, as well as truncated c-*raf* and *ret*-II oncogenes. The exogenous oncogene sequences were lost concomitant with the morphological reversion (Nakayasu et al. 1988). In contrast to this, endogenous *ras* sequences were not affected by BA treatment. Other inhibitors of poly(ADP ribose) polymerase were also able to induce the flat morphological phenotype. One of those, 1,2-benzopyrone (coumarin), prevented tumorigenesis of *ras*-transformed fibroblasts (Tseng et al. 1987). In contrast to benzamide-treated transformed cells, $p21^{ras}$ expression was not impaired. Thus, the action of the drug does neither directly nor indirectly involve the removal of the exogenous oncogene.

Melittin, an amphipathic peptide of 26 amino acids isolated from bee venom was shown to specifically select against cultured cells that express high levels of the *ras* oncogene (Sharma 1992). Cell killing by mellitin is specific for *ras* transformed cells, since nontransformed cells and cells transformed by the tyrosine kinase oncogene *ros*-1 or SV40 large T antigen were less sensitive. Acquisition of resistance to increasing concentrations of melittin is correlated with decreasing *ras* expression. Melittin was shown to be a potent activator of cellular phospholipase A_2 (PLA_2) in fibroblasts (Shier et al. 1979; Argiolas and Pisano 1983). Therefore, the authors suggest that hyperactivation of PLA_2 by melittin leads to the selective destruction of *ras*-transformed cells and that PLA_2 is an effector in the *ras* signal transduction pathway.

Masumoto et al. (1992) have screened microbial cultures for antitumor agents with "detransforming" activity. They identified a novel compound designated depudecin that inhibited the growth and induced the flat phenotype of NIH/3T3 cells doubly transformed by *ras* and *src* oncogenes. Morphological reversion was reversible. The effect of this drug on the expression of other transformed phenotypes was not reported.

An alternative strategy to diminish the concentration of the *ras* gene product is based on the introduction of antisense mRNA, hybridizing to mRNA encoding $p21^{ras}$ or a target molecule involved in *ras* signaling, of ribozymes, and antisense oligonucleotides. EJ *ras*-transformed NIH/3T3 cells were transfected with a plasmid containing an 84-bp sequence of the 5'

end of the murine c-*fos* gene controlled by the dexamethasone-inducible MMTV promoter. The partial c-*fos* sequence was in antisense orientation relative to the promoter. Expression of the antisense RNA caused a significant decrease in the amount of the c-*fos* protein expressed after serum stimulation. The *ras*-transformed cells expressing antisense-*fos* RNA continued to overexpress *ras* and retained their proliferative capacity. However, their transformed properties were partially suppressed. Thus, inhibition of c-*fos* expression prevented the transforming ability of the *ras* oncogene. It was concluded that the *fos* proto-oncogene participates in *ras*-regulated signal transduction pathways (Ledwith et al. 1990).

Ras oncogene expression was successfully reduced by introduction of hammerhead ribozymes, designed to cleave c-Ha-*ras* mRNA mutated at codon 12, into NIH/3T3 cells transformed with the same oncogene. These ribozymes inhibited focus formation by about 50% and permitted to isolate morphological revertants of *ras*-transformed cells (Koizumi et al. 1992). Mutant Ha-*ras* mRNA expression was also selectively inhibited by antisense oligonucleotides centered around the point mutation at codon 12 (Monia et al. 1992) or codon 61 (Chang et al. 1991).

5 Gene Expression Associated with the Reversion of *ras*-Transformed Phenotypes

Various groups have identified genes that are specifically expressed in phenotypic revertants (Table 2), while expression is repressed in *ras*-transformed cells. Those genes were detected by differential hybridization techniques such that cDNA libraries were constructed with mRNA from revertants and that duplicate recombinant phage plaque lifts were hybridized with radioactively labeled cDNA from either transformed precursor cells or from revertants. The insert cDNAs of differentially hybridizing clones were then used as probes on northern blots of RNAs from normal, *ras*-transformed, and revertant cell lines (Müllauer et al. 1991; Contente et al. 1990; Krzyzoziak et al. 1992). An enrichment of revertant-specific cDNA clones was achieved by subtraction cloning. Single-stranded revertant cDNA was hybridized with an excess of mRNA from *ras*-transformed cells (Hajnal et al. 1993a,b; 1994). Alternatively, the overall protein synthesis in *ras*- or *fos*-transformed cells and revertants was compared by two-dimensional gel electrophoresis (Hoemann and Zarbl 1990; Higgins and Ryan 1991).

Elevated mRNA expression of α2 (type I) collagen (Müllauer et al. 1991), gelsolin, and α-actin (Müllauer et al. 1990) was found in the revertant cell line R1 (Kuzumaki et al. 1989). Revertant cell lines derived from transformed Rat-1 cells were resistant toward transformation by v-*fos* or *ras*

Table 2. Gene expression associated with phenotypic reversion of *ras* transformation

Gene/protein	Localization of gene product
α1 (Type I) collagen	Extracellular matrix
α2 (Type I) collagen	Extracellular matrix
Type III collagen	Extracellular matrix
Fibronectin	Extracellular matrix
Lysyl oxidase (*rrg*)	Cell supernate, extracellular matrix
Plasminogen activator inhibitor I	Extracellular matrix
α-Actin	Cytoskeleton
Gelsolin	Cytoskeleton
p92-5.7 (gelsolin variant)	Cytoskeleton
Vinculin	Cytoskeleton
Tropomyosin	Cytoskeleton
Cytochrome *b*	Mitochondria
Cytochrome *c* oxidase subunit II	Mitochondria
NADH dehydrogenase 1	Mitochondria
NADH dehydrogenase 4	Mitochondria
Intracisternal A particles	Cytoplasm
RhoB	Associated with cytoskeleton
H-rev 107	Membrane fraction, cytoplasm

oncogenes and resumed the synthesis of α1(type I) and α2(I) collagen. Moreover, it was shown that α1(I) procollagen but not α2(I) procollagen was regulated at the level of transcription in Rat-1 fibroblasts, transformed cells and revertants (Hoemann and Zarbl 1990). Gelsolin is an actin binding protein. A variant form of gelsolin, p92-5.7, was identified as a newly expressed protein in the revertant cell line R1 by two-dimensional gel electrophoresis (Fujita et al. 1990). This protein was not detected in a number of normal cell lines and primary embryo fibroblasts. Flat revertants derived from *ras*-transformed NIH/3T3 cells by azatyrosine treatment specifically expressed collagen type III. In addition, fibronectin expression that was downregulated on *ras* transformation was restored in revertants (Itoh et al. 1989; Krzyzoziak et al. 1992). Genes encoding (type I) procollagens were abundantly represented in a subtracted cDNA libary specific for normal rat 208F fibroblasts prior to the introduction of a Ha-*ras* oncogene. Reexpression of different collagen genes was found in phenotypic revertants (Hajnal et al. 1993a). Elevated expression of cytoskeletal and extracellular matrix components in revertants was confirmed at the protein level. The

expression of nonmuscle tropomyosin was restored to pretransformation levels in ouabain-selected revertants of v-Ki-*ras* transformed NIH/3T3 cells (Bassin and Noda 1987). Plasminogen-activator inhibitor type I (PAI-1), an extracellular matrix-associated 52-kDa protein, was induced during the generation of the revertant phenotype. p52(PAI-1) expression was regulated at the level of mRNA abundance. A three- to fourfold increase in cytoskeletal deposition of gelsolin and vinculin (Higgins and Ryan 1991) was reported in butyrate-induced revertants of v-*ras* transformed rat kidney cells (Higgins and Ryan 1989; Ryan and Higgins 1989). This pattern of microfilament-associated proteins correlated with the revertant-specific reorganization of microfilament and focal contact formation. A similar increase in actin content was observed, whereas the abundance of intermediate filament components vimentin and lamins remained unaltered. These results suggest a role for both cytoskeletal proteins and extracellular matrix components in restructuring the normal cytoarchitecture during the process of reversion.

Contente et al. (1990) described a gene, designated *rrg* (*ras* recision gene) in the revertant cell line PR4, a persistent revertant of LTR-c-Ha-*ras* transformed NIH/3T3 cells treated with IFN-α/β. The expression of *rrg* was reduced to an undetectable level in *ras* transformed cells and incompletely restored in the revertant. Functional studies provided circumstantial evidence that *rrg* expression was sufficient to restore a more normal phenotype in *ras*-transformed NIH/3T3 cells. When persistent revertants were stably transfected with a *rrg* cDNA antisense expression vector, *rrg* mRNA was reduced in association with retransformation. Although this finding qualified *rrg* as a potential transformation suppressor gene, it is doubtful whether the inactivation of *rrg* is a necessary step for the tumorigenic conversion of a normal cell. Expression of antisense rrg mRNA in NIH/3T3 cells did not cause neoplastic transformation. The downregulation of *rrg* expression was not specific for *ras*-transformed cells. A significant decrease in *rrg* expression was also observed in v-*raf*, and v-*fes* transformed cell lines. Variable amounts of *rrg* mRNA were detected in cells harboring a SV40 early promoter–c-*myc* recombinant gene or v-*abl*, v-*sis*, and v-*mos* oncogenes.

Sequence comparisons between *rrg* cDNA and a 2672 bp cDNA of rat aorta lysyl oxidase (Trackman et al. 1990) demonstrated that *rrg* encodes lysyl oxidase (Kenyon et al. 1991). Thus, the product of *rrg* is a copper-dependent amine oxidase that catalyzes the oxidative deamination of peptidyl lysine residues in procollagen and proelastin (Trackman et al. 1990). A nonenzymatic condensation reaction forms lysine-derived cross-links in the mature extracellular matrix proteins. More than 90% of lysyl oxidase activity was found in the cell supernatant. A significant reduction of enzyme activity correlated well with the tumorigenic phenotype of *ras*-transformed NIH/3T3 cells and of rerevertants induced by antisense *rrg* expression

(Kenyon et al. 1991). Upregulation of lysyl oxidase was found in other revertant cells as well. These include revertants obtained from *ras*-transformed NIH/3T3 cells by treatment with azatyrosine (Kryzoziak et al. 1992) and IFN-γ (H. Oberhuber, R. Schäfer, B. Seliger, unpublished) as well as spontaneous morphological revertants of *ras*-transformed rat 208F cells (Hajnal et al. 1993b). The posttranslational modification of extracellular matrix precursor polypeptides by lysyl oxidase may modulate cell morphology and interactions between cells and extracellular matrix. It has been speculated that lysyl oxidase may indirectly modulate important signal transduction pathways by oxidation of membrane-bound receptors (Kenyon et al. 1991).

Four different mitochondrial genes encoding cytochrome *b*, cytochrome *c* oxidase subunit II, as well as NADH dehydrogenases 1 and 4 were specifically expressed in the flat revertant cell line R1 (Müllauer et al. 1991). Azatyrosine-induced revertants derived from *ras*-transformed human mammary epithelial cells showed an increased level of K-*rev*-1 mRNA expression (Kyprianou and Taylor-Papadimitriou 1992). Therefore, the reversion of transformed human mammary epithelial cells may be mediated by an elevated level of K-*rev*-1 expression (see next section). Azatyrosine-induced revertants expressed *rhoB* and sequences corresponding to that of murine retrovirus-like intracisternal particles (IAP). IAP-related cDNAs represented about 50% of the clones specific for the reverted state and identified by differential cDNA hybridization (Kryzosiak et al. 1992). Rho, a *ras*-related GTP binding protein, rapidly stimulated stress fiber and focal adhesion formation after microinjection into serum-starved Swiss 3T3 cells (Paterson 1990; Ridley and Hall 1992). These findings suggest that Rho is an essential protein for the coordinated assembly of focal adhesions and actin stress fibers in growth-stimulated cells. Dysfunction of *rho* by downregulation of its expression may at least partially be responsible for the deformation of stress fibers on oncogene-mediated transformation. The reestablishment of *rho* expression may significantly contribute to those events that mediate the normal morphology of revertants.

By subtraction cloning Hajnal et al. (1994) have isolated a novel gene, designated H-*rev*107, which is specifically expressed in a revertant clone obtained from Ha-*ras* transformed rat 208F cells. Rat cells transformed by Ha-*ras* or v-*src* oncogenes do not contain detectable amounts of H-*rev107* mRNA, whereas logarithmically growing fibroblastic cell lines such as NIH/3T3 and 208F express very little. In contrast to this, H-*rev107* mRNA was abundant in *ras*-transformation-resistant cell lines REF-52 and EK-3. Introduction of the adenovirus E1A oncogene into REF-52 cells, which abolishes the resistance toward *ras* transforming activity (Franza et al. 1986), repressed the H-*rev*107 gene. The function of H-*rev*107 is still un-

known. The protein product of the H-*rev*107 gene exhibits an apparent molecular mass of 18,000 and has no sequence similarity to known proteins. $P18^{H\text{-}rev107}$ has a hydrophobic C-terminal stretch of amino acids. Two forms of the protein were distinguishible in cellular extracts of REF-52 cells by SDS gel electrophoresis. The faster migrating form was localized in the cytoplasm, the other was present predominantly in the membrane fraction. When normal rat 208F cells were subjected to density-dependent growth arrest, membrane-associated $p18^{H\text{-}rev107}$ was accumulated. Growth arrest induced by serum starvation did not induce expression of H-*rev*107 (Hajnal et al. 1994).

6 *ras* Transformation Suppressor Genes

In the previous section several genes were discussed that appear to be involved in the maintenance of the reverted state in derivatives of *ras*-transformed cells (Table 2). It is desirable to know which genes have the capability to induce phenotypic reversion. Functional assays have been instrumental in identifying transforming genes. A similar approach is difficult to design for genes whose function is to constrain growth since their expression does not confer a growth advantage on cells. Following transfection of genomic DNA or of complementary DNA from normal cells into *ras*-transformed recipient cells, revertant clones were obtained by drug selection and/or morphological selection. DNA sequences were identified capable of counteracting *ras*-mediated transformation (Schäfer et al. 1988; Noda et al. 1989; Noda 1990; Cutler et al. 1992). Genomic DNA prepared from human placental cells was transfected into rat FE-8 cells, a neoplastically transformed derivative of 208F cells expressing an activated human Ha-*ras* oncogene (Schäfer et al. 1988). The population of transfected FE-8 cells, representing the entire donor genome in about 6 000 transfectant clones, was subjected to selection with ouabain. This drug was used to selectively eliminate those transfectants that continued to express the neoplastic phenotype. A few revertant clones survived ouabain-selection and continued to express the reverted phenotype. In a subsequent transfection cycle, the reverted phenotype was passaged onto FE-8 cells. A recombinant DNA library was constructed from the DNA of a secondary transfectant. Screening this library with a repetitive human Alu probe identified one recombinant clone that carried the suppressing activity. However, the mechanism of suppression is still obscure, since sequence analysis of cloned transfected human DNA revealed an extensive structural DNA rearrangement and no open reading frame. Possibly, the suppressing activity is mediated by transcription of DNA sequences harboring a protein binding site (M. Kiess and

Table 3. Suppressors of *ras* oncogene induced transformation

Gene	Subcellular localization and biochemical properties of gene product	Proposed cellular function
Krev-1/rap1A/smg 21	Membrane, GTP-binding, GTP-hydrolysis	Signal transduction oxygen radical production
rsp-1	?	Signal transduction
GAP	Cytoplasmic[a], activation of GTP-hydrolysis	Signal transduction
NF-1 (neurofibromin)	Membrane (cytoplasmic)[b], activation of GTP-hydrolysis	Signal transduction
H-*ras*	Membrane, GTP-binding, GTP-hydrolysis	Signal transduction
Thy-1	Cell surface, glycoprotein	Cell-cell recognition
gas-1	Transmembrane protein	Cell cycle control
p53	Nuclear phosphoprotein	Transcriptional regulation, growth control

[a] May translocate to membrane.
[b] Membrane-associated in many tissues, cytoplasmic in brain.

R. Schäfer, unpublished observations). The two other groups have used cDNA expression libraries for the transfer of suppressing activity into *ras*-transformed cells and been able to identify two functionally active genes, designated *Krev-1* (Kitayama et al. 1989) and *rsp-1* (Cutler et al. 1992). A list of genes capable of suppressing transformation by mutated Ras is shown in Table 3.

6.1 *Krev-1/rap1A*

6.1.1 Identification by Functional Assay

Seven flat revertant cell lines were isolated from DT, a Kirsten murine sarcoma virus transformed NIH 3T3 cell line following transfection of a human fibroblast cDNA library (Noda et al. 1989; Kitayama et al. 1989). The human foreskin fibroblast expression library was constructed in the eukaryotic expression vector pcD2 harboring the *neo* gene as a selectable marker (Chen and Okayama 1987). The transfected cDNA was recovered from one revertant clone, R16, in the following way: *SalI*-digested DNA from R16 cells was circularized by ligation and transformed into competent *Escherichia coli*. Although the *neo* gene is controlled by a eukaryotic pro-

moter in pcD2, it is able to confer weak kanamycin resistance on bacteria. Several kanamyin-resistant plasmid clones were transfected into DT cells. One clone, pKrev-1, conferred the revertant phenotype onto v-Ki-*ras* transformed cells (Kitayama et al. 1989). Morphological reversion of DT cells requires high *Krev* -1 expression. *Krev*-1 cDNA encodes a 184 amino acid open reading frame, corresponding to a protein with a calculated molecular mass of 21 kDa. This open reading frame shares strong structural similarity with *ras* proteins. The similarities are confined to the β-phosphoryl group and guanine binding domains of the Ha-*ras* protein. Moreover, the effector domain in the Ha-*ras* (amino acid residues 32–44) and the *Krev*-1 proteins is identical. As *ras* proteins, the predicted *Krev*-1 protein has a CAAX consensus sequence at its extreme carboxy-terminal end (C, cysteine residue; A, aliphatic amino acid; X, any amino acid residue). In the Ha-*ras* protein, this amino acid sequence is necessary for membrane attachment and transforming activity. Protein domains with less similarity between *Krev*-1 and *ras* are the residues 59–63, known to harbor point mutations in oncogenically activated *ras* proteins, residues 72–85, found in high molecular weight G-proteins, and residues 152–164 that are essential for Ha-*ras* transforming activity. The α-helical region located at the carboxy-terminal end of *Krev*-1 is shorter than those of *ras* proteins. The *Krev*-1 gene is expressed in many tissues including brain, thymus, lung, spleen, colon, and ovary.

One year before the identification of *Krev*-1, three genes, designated *rap1A*, *rap1B*, and *rap2*, were isolated from a human cDNA library by low-stringency hybridization with the *Drosophila Dras*3 gene (Pizon et al. 1988a,b). In the same year a novel small GTP binding protein was purified from bovine brain crude membranes and its cDNA cloned from a bovine brain cDNA library. The protein was designated smg p21 (Kawata et al. 1988). While *rap1A* is identical to *Krev*-1, the smg p21 gene is identical to *rap1B*. The *rap1A* proteinwas independently isolated from human neutrophils (Bokoch et al. 1988; Quilliam et al. 1990). The product of the *Krev*-1/*rap1A* gene was copurified as a component of the superoxide generating system from human neutrophils (Quinn et al. 1989). The *rap1B* protein was also purified from human platelets (Ohmori et al. 1989; Matsui et al. 1990) and bovine aorta smooth muscle (Kawata et al. 1989; 1991). A close relative of the *rap*2 gene, designated *rap2B*, was isolated from a human platelet cDNA library (Farrell et al. 1990; Ohmstede et al. 1990; Lerosey et al. 1991). The *rap*1 and *rap*2 proteins exhibit 70% identity at the amino acid level while *rap1A*/*rap1B* and *rap2A* and *rap2B* proteins are 95% and 90% identical, respectively (for review see Bokoch 1993).

The frequency of morphological reversion of Ki-*ras* transformed DT cells induced by overexpression of *Krev*-1 was rather low (2%–5% of total transfectants; Kitayama et al. 1989). Site-directed mutagenesis of the *Krev*-1

cDNA was performed to further investigate the biological activity of the *Krev*-1 gene. Point mutations in codon 38 resulting in the substitution of Asp by Ala or Asn inhibited the ability of the gene to cause phenotypic reversion. The reversion frequency was enhanced between two- and fivefold by amino acid exchanges such as Gly (codon 12) to Val and Gln (codon 63) to Glu (Kitayama et al. 1990). In addition to suppressing the transformed properties of DT cells, these *Krev*-1 mutants showed strong tumor suppressor activities in HT 1080 fibrosarcoma cells harboring the activated N-*ras* gene. In another study, chimeric *Krev*-1/Ha-*ras* genes were assayed for transforming or suppressing activity (Zhang et al. 1990). The critical sequences for transformation by v-Ha-*ras* or suppression by *Krev*-1 were localized to the NH_2-terminal 54 amino acids. Amino acids 32–44 are identical in *Krev*-1 and *ras* (Kitayama et al. 1989). The *ras*-specific and *Krev*-1 specific amino acids immediately surrounding these amino acid residues, particularly residues 26, 27, 30, 31, and 45 determined whether the protein has transforming activity or acts as a transformation suppressor (Zhang et al. 1990; Marshall et al. 1991; Nur-e-Kamal et al. 1992). This region of $p21^{ras}$ represents the effector binding domain (Barbacid 1987; Grand and Owen 1991), indicating that *Krev*-1 suppresses *ras*-mediated transformation by competing with the oncoprotein for a target molecule (or molecules).

The suppressing effect of *Krev*-1 was also assessed in another human tumor cell line. Cell clones expressing exogenous *Krev*-1 mRNA were derived from the anaplastic lung carcinoma cell line Calu-6. In contrast to parental cells, revertant lines had a flat or squamous morphology and proliferated continuously, independent of a low serum concentration in the medium. After subcutaneous inoculation into nude mice, the revertant cells formed tumors albeit with a reduced growth rate. In a tracheal transplantation assay, the invasive properties of parental cells and revertant lines were compared. However, a consistent loss of invasiveness was not found in different revertant cell lines. Only one revertant line was able to metastasize spontaneously, while parental cells exhibited a high metastatic potential (Zucker et al. 1992). In view of these weak effects on tumorigenicity and metastactic potential, it was of interest to find out whether *Krev*-1 may prevent neoplastic transformation in normal cells when expressed with an oncogene. Preneoplastic Rat-2 cells were cotransfected with a low amount of plasmid DNA, in which the polyomavirus early promoter controls middle T (mT) antigen expression, and with increasing amounts of p*Krev*-1 DNA (Jelinek and Hassell 1992). mT antigen-mediated morphological transformation of Rat-2 cells was inhibited in a dose-dependent manner. Inhibition was about 85% of the control at the highest dose of p*Krev*-1 DNA used. This inhibitory effect appeared to be specific, since morphological transformation by SV40 large T antigen or the frequency of puromycin-resistant colonies

was unaffected by *Krev*-1 DNA cotransfer in control experiments. Moreover, the same authors isolated 16 morphological revertant cell lines after transfection of p*Krev*-1 into mT-antigen transformed cells. Morphological revertants represented only about 1% of the total transfectants, thus confirming the rather weak activity of the transformation suppressor described earlier (Kitayama et al. 1989). One exceptional clone had lost the oncogene. The other 15 clones differed from their transformed precursor cell line in their reduced growth rates and low capacity to form colonies in semisolid agar medium. The ability to form colonies in agar medium was inversely proportional to the amount of *Krev*-1 mRNA and protein expressed. The results of this study confirmed an earlier result (Smith et al. 1986) that $p21^{ras}$, which is counteracted by *Krev*-1, lies downstream of mt antigen and pp60 c-*src* in the same mitogenic signal transduction pathway (Jelinek and Hassell 1992).

Although Rap2 proteins contain an effector domain identical to Ras, they do not suppress Ras-mediated transformation (Schweighoffer et al. 1990; Jimenez et al. 1991). The intrinsic GTPase activity of Rap2A was not stimulated by rasGAP and there was no competition with Ras for interaction with *ras* GTPase activating protein (GAP; Lerosey et al. 1992).

If the *Krev*-1 gene plays an important role in the multistep process of tumorigenesis, one would expect to find tumors in which its structure or expression is altered. Therefore, the expression of *Krev*-1 was investigated in tumorigenic cells. *Krev*-1 related transcripts were abundant in colonic cells (Kitayama et al. 1989). The locus of *Krev*-1/*rap1A* has been mapped to human chromosome 1p12-p13. The *rap1B* and *rap*2 genes have been assigned to bands 12q14 and 13q34 of the same chromosome, respectively (Rousseau-Merck et al. 1990). Both structural and numerical abberrations of the short arm of chromosome 1 are frequent in tumors, including colorectal tumors (Mitelman 1990; Muleris et al. 1990). Moreover, oncogenic activation of Ki-*ras* genes is frequent in colorectal cancer (Vogelstein et al. 1988). These findings prompted an investigation of the *Krev*-1/*rap1A* locus in human colon cancer. Young et al. (1992) identified a *Krev*-1-specific *Bcl*I restriction fragment length polymorphism (RFLP) that permitted to search for allelic deletions by comparison of the constitutional chromosome content and of tumor tissue. Only one out of 18 tumors from informative cancer patients showed allelic loss at this locus, suggesting that loss of heterozygosity at the *Krev*-1 locus is a rare event in colorectal tumors. Culine et al. (1989) have reported northern blot analysis of the three *rap* genes in 41 primary human tumors, including 13 lymphomas, 14 carcinomas, 6 sarcomas, various tumors of the nervous system, and germinal neoplasms of the testis. A significant decrease in the expression of the *rap1A* gene was observed in one salivary gland adenocarcinoma and in the sarcomas tested

with the exception of the leiomyosarcoma. In contrast, *rap1B* was highly and uniformly transcribed in nearly all normal and malignant tissues analyzed. Usually, detection of *rap2* expression was only possible in polyadenylated RNA. The *rap2* expression was absent in sarcoma poly(A) RNA samples. The tumors were chosen on the basis of a low incidence of *ras* mutations. The significance of *rap* gene downregulation in human multistep carcinogenesis is not clear.

In an attempt to investigate the potential role of *rap1A* and *rap1B* genes in mammary tumorigenesis in rats, mammary tissues from tumor-susceptible Sprague-Dawley and tumor-resistant Copenhagen rats were screened for deregulation of *rap* gene transcription and for mutations. However, differences between those two inbred rat strains were not found (Hong et al. 1990; Hsu and Gould 1991).

6.1.2 Mechanism of Interference with ras Function

The greatest divergence in amino acid sequence between Ras and Rap1A occurs at the C-terminus. Krev-1/Rap1A is posttranslationally modified by a C_{20} geranylgeranyl group at Cys-181. The last three amino acids are proteolytically cleaved off and the terminal prenylated cysteine is carboxymethylated (Buss et al. 1991). Ras contains a C_{15} farnesyl at its terminal cysteine residue. As shown by functional analysis of Ras/Rap1A proteins, farnesyl and geranylgeranyl moieties are mutually interchangeable for transforming and suppressing activity, respectively (Buss et al. 1991; Cox et al. 1992). Fractionation of cell extracts has shown that Rap1 and Ras were not associated (Grand and Owen 1991; Beranger et al. 1991, Kim et al. 1990). In Rat-1 fibroblasts and Hep2 epidermoid carcinoma cells, Rap1 was associated with a Golgi-like structure, while Ras was found in the cytoplasmic membrane fraction (Beranger et al. 1991). Association of Rap1 with both the plasma membrane and granular structures was, however, found in neutrophils (Maridonneau-Parini and de Gunzburg 1992; Quinn et al. 1992). Rap1A was shown to be phosphorylated at Ser-180 by cyclic AMP-dependent protein kinase in vitro. (Kawata et al. 1989; Lapetina et al. 1989; Quilliam et al. 1991). $p21^{rap1A}$ appears to be identical to thrombolamban, a 22-kDa protein, which serves as a major substrate for protein kinase A in platelets (Fischer and White 1987). It has been speculated that Rap1A is involved in the release of Ca^{2+}, and that phosphorylation of this protein by c-AMP activated protein kinase C inhibits this function (Kawata et al. 1989; Lapetina et al. 1989). Phosphorylation upon differentiation was demonstrated in intact HL-60 cells (Quilliam et al. 1991). Phosphorylation of Rap1A did not affect guanine nucleotide binding and hydrolytic activity.

The identity of $p21^{ras}$ and $p21^{rap1A}$ proteins in the effector region suggested that GAP, a known effector of *ras* action (for review see Downward 1992; Hall 1992) also interacts with the product of *Krev*-1. Therefore the ability of GAP to interact with Rap1A-p21 was tested in vitro. Rap1A bound tightly to GAP and effectively inhibited GAP-mediated Ras-GTPase activity. The binding of GAP to Rap1A was GTP dependent. The affinity of Rap1A-GTP for rasGAP was more than 50-fold greater than that of Ras-GTP. Thus, Rap1A may compete with Ras for its downstream effector. The GTPase activity of Rap1A was not stimulated by rasGAP, suggesting the Rap1A/rasGAP complex was catalytically nonfunctional (Frech et al. 1990; Hata et al. 1990).

Rap1A does not only associate with rasGAP but also with a GTPase activating protein that is specific for Rap1 and shows no significant similarity to rasGAP (Rubinfeld et al. 1991, 1992). Initially, two smg p21 GAP proteins were isolated from bovine brain and shown to be distinct from p21*ras* GAP and $p20^{rho}$ GAP (Kikuchi et al. 1989). Later, a membrane-associated rapGAP protein was purified from differentiated promyelocytic HL60 cells (Polakis et al. 1991). The gene encoding rapGAP was isolated from a human brain cDNA library (Rubinfeld et al. 1991). Apart from the sequence difference, rap1GAP and rasGAP can be distinguished by their mode of interaction with the GTP binding proteins Ras and Rap1: GTP hydrolysis is brought about by different mechanisms. Glu-61 is essential for GTP hydrolysis by Ras. The same amino acid residue is not critical for this reaction by Rap1. When Thr-61 of $p21^{rap1A}$ was mutated to glutamine, rasGAP could accelerate the rate of GTP hydrolysis (Hart and Marshall 1990). Binding of Rap1 to its specific GAP occurs with both GTP-bound and GDP-bound forms, while Rap1A binding to rasGAP is GTP-dependent. It was proposed that the function of Rap1 depends upon its interaction with both rasGAP and rap1GAP (Rubinfeld et al. 1991). First, binding of Rap1 to rasGAP may inhibit the interaction of Ras and rasGAP (Frech et al. 1990; Hata et al. 1990). Second, upon binding of Rap1 to its specific GAP, bound GTP is hydrolyzed and the protein switched into its GDP-bound state. Since the binding of Rap1 to rasGAP is dependent upon GTP, the ability of Rap1 to antagonize Ras (i.e., to compete for rasGAP binding with p21*ras*) would be regulated by interaction with its own specific GAP. The binding of $p21^{ras}$ to rasGAP is thought to result in a growth-promoting signal. When Rap1-GTP binds to the same downstream effector, this growth signal would be disrupted (for review see Hall 1990; McCormick 1989). It is assumed that Rap1 would be in the GDP-bound form in growing cells, due to its interaction with rap1GAP, and would be unable to perturb Ras function. It cannot be excluded however, that Rap1 signaling is transmitted via a separate pathway which also requires the GTP-bound form of the protein (Rubinfeld

et al. 1991). The issue is further complicated by the fact that rasGAP may act as an upstream regulator of Ras rather than as a downstream target (Hall 1990).

A GDP/GTP dissociation stimulator, Rap1-GDS, has been identified and its cDNA been cloned (Yamamoto et al. 1990; Kaibuchi et al. 1991; Kikuchi et al. 1992). The 53-kDa protein is a relative of the yeast CDC25 and SCD25 proteins and is able to catalyze GDP/GTP exchange for Rap1A, Rap1B, Ki-Ras, Rho A, and Rac1 (Mizuno et al. 1991; Kotani et al. 1992). GTP binding of Rap1B may be regulated by binding to certain phospholipids via its C-terminal domain and by cyclic AMP dependent kinase (see Bokoch 1993).

6.1.3 Multiple Biological Activities

Rap1A was shown to modulate different physiological processes which involve signal transduction mediated by Ras. These processes include the short-term stimulation of DNA synthesis in Swiss 3T3 cells, transcription of the immediate early gene c-*fos*, opening of K^+ channels in atrial membranes, mitotic maturation in *Xenopus*, and photoreceptor development in *Drosophila*.

The cellular level $p21^{Krev\text{-}1/rap1}$ was elevated to about 120-to 150-fold of the endogenous level by microinjection into Swiss 3T3 cells of the protein synthesized in *E. coli* or *Spodoptera frugiperda*. Both $p21^{Krev\text{-}1}$ and $p21^{Ki\text{-}ras}$ (Val-12) induced DNA synthesis when in their GTP-bound form. Their action differed in that Ras induced membrane ruffling irrespective of the presence or absence of insulin, whereas the GTP-bound form of Rap1A required insulin. Ras microinjection also resulted in the decrease of stress fibers and rounding up of cells. In contrast, Rap1A did not cause morphological transformation (Yoshida et al. 1992).

The effect of *Krev*-1/*rap*1A cDNA on the c-*fos* promoter/enhancer linked to the luciferase reporter gene (c-*fos* luciferase) was examined by transient transfection into Cos cells (Sakoda et al. 1992). While the basal expression of the reporter gene in NIH/3T3 cells was rather low, c-*fos* luciferase expression was elevated in NIH/3T3 cells constitutively expressing a mutant Ki-*ras* (Val-12) or activated c-*raf*-1 kinase. Treatment with 12-tetradecanoyl phorbol 13-acetate (phorbol ester, TPA), platelet-derived growth factor (PDGF), or dibutyryl cyclic AMP (Bt2cAMP) also resulted in enhanced c-*fos* luciferase expression. Overexpression of *rap*1A and *rap*1B cDNAs inhibited the stimulation of c-*fos* luciferase mediated by $p21^{ras}$, PDGF, or TPA. However, c-raf-1 kinase or Bt2cAMP stimulation of the reporter gene was not affected. Thus, Rap proteins inhibited the signal transduction pathway from the PDGF receptor via protein kinase C and $p21^{ras}$ effectors to the

c-*fos* promoter/enhancer, whereas signaling from *raf* kinase and cAMP-dependent protein kinase was not impaired.

Rap1A was able to inhibit the concerted negative effects of Ras and Ras-GAP on opening of M_2 muscarinic receptor-regulated K^+ channels of atrial membranes (Yatani et al. 1990, 1991). Particularly, an intact Rap1A effector domain was required for antagonistic function and Rap1A did not block the inhibition of channel opening by a form of GAP that was independent of Ras (Yatani et al. 1991).

Xenopus laevis oocytes (stage 6) can be induced to proceed through mitotic maturation, also called germinal vesicle breakdown (GVBD), by progesterone, insulin, or insulin-like growth factor 1 (IGF-1). GVBD is blocked after microinjection of antibodies against the *ras* gene product (Korn et al. 1987) and stimulated oncogenic Ha-*ras* proteins, such as the Val-12 mutant (Birchmeier et al. 1985). GVDB induced by Ras was effectively blocked by coinjection of equimolar amounts of the Rap1B. This inhibition of *ras*-induced GVBD was in turn lifted when IGF-1 was added to the incubation medium (Campa et al. 1991).

The human and *Drosophila rap* genes are extremely similar (Pizon et al. 1988). The Phe-157 residue in *Drosophila rap*1 corresponds to Phe-158 in human Rap1A and Phe-156 in human Ha-Ras. This amino acid residue is one of only five residues outside the GTP binding region which are conserved in every member of the *ras* superfamily. Substitution of Leu for Phe in *Drosophila Rap*1 causes a gain-of-function mutation resulting in the absence of an R7 photoreceptor cell in many omnatidia (*roughened* mutation; Hariharan et al. 1991). The correct diffentiation of R7 cells involves a signal transduction pathway, in which the product of the sevenless receptor kinase gene (Hafen et al. 1987) and the *ras*1 gene are important components (Simon et al. 1991). It is possible that Rap1 antagonizes Ras1 in the process of R7 diffentiation. Loss-of-function mutations of *Drosophila rap*1 were lethal (Hariharan et al. 1991).

Rap1A interacts with the phagocyte NADPH oxidase/cytochrome *b* system. Neutrophils and other phagocytic cells utilize a multicomponent enzyme complex for the reduction of molecular oxygen to form superoxide anion and other toxic oxygen metabolites in response to contact with opsinized micro-organisms ("respiratory burst"). This enzyme complex consists of at least four proteins including cytochrome b_{558} (see Bokoch 1993 for a review). The first indication that Rap1A might modulate this enzyme complex derived from a report by Quinn et al. (1989) who copurified a protein of relative molecular mass 22 000 with cytochrome *b*. The sequence of the cDNA was identical to the *Krev*-1 sequence. The possibility of a direct association of *Krev*-1/*rap*1 protein with cytochrome *b* was confirmed by immunoaffinity purification methods with matrices conjugated with anticytochrome and anti-Ras antibodies. The elution pattern suggested an associ-

ation of immunopurified neutrophil cytochrome *b* and the *ras*-related protein. Complexes between the two proteins were also detected in vitro with purified cytochrome *b* and recombinant Rap1A expressed in baculovirus-infected Sf9 cells (Bokoch et al. 1991). The guanosine-5'-O-(3-thiotriphosphate; GTP-γS) bound form of Rap1A bound more tightly to cytochrome *b* than did the GDP-bound form. Complex formation between Rap1A and cytochrome *b* was inhibited by phosphorylation of Rap1A by protein kinase A (Bokoch et al. 1991). The significance of the interaction of Rap1A with NADPH oxidase-associated cytochrome *b* is not yet understood (Bokoch 1993).

6.2 *rsp*-1

An expression cloning assay, similar to the one described by Noda et al. (1989) was used to isolate a novel cDNA, referred to as *rsp*-1, which is able to suppress the v-*ras* transformed phenotype (Cutler et al. 1992). The expression library was constructed on the basis of RNA from the revertant cell line CHP9CJ (Yanagihara et al. 1990; see above). The v-*ras*-transformed NIH/3T3 cell line DT was used as recipient. After selection in G 418 and treatment of transfectants with the aminoglycoside ouabain (Noda et al. 1983), flat revertants were isolated. Several cDNAs isolated from one primary transfectant clone were able to confer the flat phenotype onto DT cells at a moderate frequency of 10%–20%. Full-length *rsp*-1 cDNA contains a 831-bp open reading frame encoding a 277 amino acid protein. The mouse and human *rsp*-1 genes are highly conserved. A major transcript of 1.7 kb was detected in NIH/3T3 cells, DT cells, and several oncogene transformants. The *rsp*-1 coding sequence was cloned into a retroviral vector. DT cells were infected with the recombinant vector and the ability to suppress soft agar colony formation was assessed. Overexpression of *rsp*-1 resulted in a 25%–75% reduction in colony formation. *Rsp*-1 cDNA controlled by an inducible promoter was able to revert the anchorage independent phenotype of Ki-MuSV-infected NOG8 mouse mammary epithelial cells. *Rsp*-1-infected NIH/3T3 cells grew more slowly than vector control cell lines suggesting a negative or toxic effect on the growth of NIH/3T3 cells. One *rsp*-1-infected cell line exhibited resistance toward retroviruses containing v-Ki-*ras* and v-Ha-*ras* oncogenes, but were transformed by v-*src* and v-*mos* oncogenes.

The predicted *rsp*-1 protein contains several leucine-based repeats homologous to similar repeats found in the regulatory region of yeast adenylate cyclase. In *S. cerevisiae* this region of the protein is necessary for regulation of the cyclase by Ras (Colicelli et al. 1990; Field et al. 1990; Suzuki et al. 1990). Since Ras does not regulate adenylate cyclase in higher eukaryotic

cells, Cutler and colleagues (1992) have suggested that *rsp*-1 is not a component of the adenyl cyclase pathway in spite of its structural similarity to the yeast protein. Rather, they proposed that *rsp*-1 either interferes with a downstream target molecule in the Ras-signaling pathway or may represent a downstream target itself. The normal cellular function of *rsp*-1 is still unknown.

6.3 *GAP*

Xenopus oocytes contain an enzymatic activity which stimulates the rate of hydrolysis of GTP bound to microinjected $p21^{ras}$. The responsible enzyme was designated GTPase activating protein, or GAP (Trahey and McCormick 1987). The corresponding gene was cloned from bovine brain (Vogel et al. 1988) and human placenta (Trahey et al. 1988). The protein product of the GAP gene exhibits a molecular mass of 120 kDa. The sequence contains two SH2 (*src* homology) regions and one SH3 motif. SH2 domains mediate protein-protein interactions by binding to phosphotyrosine containing sequences (Cantley et al. 1991). Although $p120^{GAP}$ binds to wild-type and mutant $p21^{ras}$, it only stimulates GTP hydrolysis on normal $p21^{ras}$ (Vogel et al. 1988). Several lines of evidence have suggested that $p120^{GAP}$ may act either as an upstream regulator of $p21^{ras}$ or as a downstream effector molecule in the *ras*-mediated signal transduction pathway (for review see Downward 1992; Hall 1992). The addition of purified $p120^{GAP}$ or $p21^{ras}$ to isolated muscle cell membranes inhibited the G-protein-mediated coupling of K^+ channels to muscarinic cholinergic receptors as measured by patch clamping. When neutralizing anti-Ras antibodies were added, inhibition by $p120^{GAP}$ was lifted. Conversely, an antibody to GAP inhibited the effect of Ras, suggesting that Ras and GAP act together (Yatani et al 1990; Martin et al. 1992;). The role of $p120^{GAP}$ as a negative regulator of Ras function is suggested by its suppressing effects on oncogene-transformed cells (see below). There are a number of ways by which $p120^{GAP}$ can be regulated or modified. Regulatory mechanisms include phosphorylation on tyrosines by receptor and nonreceptor tyrosine kinases, complex formation with activated receptors such as PDGF receptor, interactions with two other phosphoproteins of 62 kDa and 190 kDa and certain lipids (Molloy et al. 1989, 1992; Ellis et al. 1990; Tsai et al. 1990; for review see Downward 1992).

The role of GAP in *ras*-mediated neoplastic transformation was investigated by cotransfection into NIH/3T3 cells of normal or mutant *ras* genes placed under the control of a murine retroviral LTR and a human GAP cDNA clone (Zhang et al. 1990). Transfection of a three to sixfold excess of GAP cDNA resulted in inhibition of focus formation (average inhibition

84%). In contrast, v-*ras* mediated transformation was not significantly affected by GAP cotransfection (average inhibition 13%). Morphological revertants were isolated following transfection of GAP cDNA into c-*ras* transformed cell lines. The level of exogenous GAP protein, rather than that of $p21^{ras}$ correlated with the morphological reversion of *ras* transformed NIH/3T3 cells. The authors concluded that GAP is an upstream regulator of *ras,* exerting its effect by reducing the level of active GTP-bound Ras protein (Zhang et al. 1990). Similar effects of GAP were reported for cells transformed by genes which appear to require functional Ras for exerting their phenotypic effects. Overexpression of translation initiation factor eIF-4E was shown to result in tumorigenic transformation of rodent fibroblasts. $p21^{ras}$ is involved in this process (Lazaris-Karatzas et al. 1992). Forced overexpression of GAP to a six to eight times elevated level compared to control eIF-4E transformed cells resulted in a flat morphology, loss of anchorage-independent growth and tumorigenicity in nude mice. At the same time, the levels of GTP-bound Ras were diminished to those found in immortalized nontumorigenic rat embryo fibroblasts. Two groups have reported that GAP inhibits neoplastic transformation by c-*src* or v-*src* (DeClue et al. 1991; Nori et al. 1991). *Src*-mediated transformation is dependent on endogenous Ras activity (Smith et al. 1986). Focus formation was reduced by about 80% when a plasmid encoding full-length GAP was cotransfected with the c-*src* oncogene. The suppressing activity of molecular clones encoding only the N-terminal amino acids 1–987 or C-terminal amino acids 685–1047 of GAP was analyzed in the same transformation system. Inhibition of c-*src* induced morphological transformation was even enhanced by the C-terminal GAP clone, while the N-terminal clone was inactive. Since the N-terminal GAP clone lacked part of the catalytic domain required for GTPase stimulation of $p21^{ras}$, it was concluded that the mechanism of inhibition is due to the catalytic downregulation of Ras by GAP. All revertants had unaltered levels of $pp60^{src}$ expression or of protein-tyrosine phosphorylation (De Clue et al. 1991). GAP-overexpressing NIH/3T3 cells were infected with a v-*src* mutant virus that is temperature-sensitive for transformation. Afterwards, the dependence of transformation on the *src* oncogene of control cells and of GAP-overexpressing cells was assayed at the permissive (35.5°C) and the restrictive (39.5°C) temperatures. The control cells converted to morphological transformants when grown at the permissive temperature and remained normal at the restrictive temperature, while GAP-overexpressing cells retained their normal flat morphology at either 35.5 or 39.5°C (Nori et al. 1991).

Further evidence for the function of rasGAP as a negative regulator of Ras was obtained from experiments using expression vectors encoding plasma membrane-targeted rasGAP (Huang et al. 1993). To achieve association of

GAP with the plasma membrane, Ras C-terminal motifs sufficient for this localization were cloned onto the C-terminus of rasGAP. Coexpression of oncogenic *ras* and the membrane-targeted GAP in NIH/3T3 cells resulted in suppression of transformation. In addition, proliferation of untransformed NIH/3T3 cells was abrogated in the absence of the oncogene.

6.4 *NF*-1 (Neurofibromin)

Several lines of evidence have indicated that the genetic locus associated with von Recklinghausen neurofibromatosis type 1 (NF-1) harbors a tumor suppressor gene. The disease primarily affects tissues derived from the neural crest. Diagnostic features of NF-1 include pigmented skin patches (cafe au lait spots), skin neurofibromas containing multiple cell types such as Schwann's cells and axons, as well as Lisch nodules, a developmental abnormality of the iris. Less common features of NF-1 are malignant tumors of the central and peripheral nervous systems (for review see Ponder 1990). The NF-1 locus was assigned to the proximal long arm of human chromosome 17 by linkage analysis (Barker et al. 1987; Seizinger et al. 1987; Goldgar et al. 1989) and by physical mapping (Fountain et al. 1989; O'Connell et al. 1989; Yagle et al. 1990). The putative gene was localized to a DNA region comprising a few hundred kilobases. DNA samples of patients carrying translocations in the chromosomal region 17q11.2 were instrumental in the identification of the NF-1 gene that was reported independently by three groups. Viskochil et al. (1990) used a DNA sequence conserved in the human and rodent genomes that spanned a translocation breakpoint to identify a cDNA clone specific for a large 11-kb mRNA. In the same way, Cawthon et al. (1990) identified cDNA clones from the translocation breakoint region and a corresponding genomic sequence. Once the boundaries of introns and exons were identified, oligonucleotide primers were synthesized and used to compare amplified exons from normal donors and NF-1 patients by single-strand conformation polymorphism analysis. DNA fragments exhibiting an altered electrophoretic mobility were sequenced and searched for mutations. Nucleotide exchanges resulting in amino acid substitutions were identified in NF-1 patients, suggesting that the gene located at the translocation breakpoint was indeed the NF-1 gene. Using chromosome jumping techniques and recombinant yeast artificial chromosomes, Wallace et al. (1990) identified an approximately 13-kb mRNA transcript from the chromosomal region that is translocated in NF-1 patients. The gene was shown to be interrupted by the t(1;17) translocation. In addition, an insertion into the gene was identified in another patient resulting in an abberrant mRNA transcript. Cloning of the complete coding region of the NF-1 transcript

revealed an open reading frame of 2818 amino acids. The gene spans approximately 300 kb and is organized into at least 30 exons (Marchuk et al. 1992). Several other genes were embedded in the region spanned by the novel gene and transcribed from the opposite DNA strand. Based on the mutational analysis, those genes were excluded as candidates for the NF-1 gene (Wallace et al. 1990; Xu et al. 1990; Cawthon et al. 1990, 1991).

6.4.1 Functional Relationship of NF-1 and Mammalian GAP

While the sequence of partial NF-1 cDNA clones initially did not reveal any similarities to known proteins, identification of additional 5' sequences permitted detection of a relevant protein domain. A sequence of 360 amino acid residues of the predicted polypeptide showed significant similarity to the C-terminal catalytic domain of human and bovine GAP proteins (Xu et al. 1990) and of the yeast IRA1 ("inhibitor of RAS") product (Buchberg et al. 1990). The overall sequence conservation of 23% between human NF-1 and yeast IRA1 is quite remarkable in that it extends over a region of more than 1500 amino acids. Later it was shown by both genetic and biochemical assays that the product of the *NF-1* gene interacts with $p21^{ras}$. A fragment of the NF-1 cDNA encoding the GAP-related domain (NF-1 GRD) was cloned in *Baculovirus* transfer or yeast expression vectors and expressed in Sf9 insect cells or in yeast, respectively. The protein was purified and assayed for its ability to stimulate human $p21^{N\text{-}ras}$ GTPase activity. GTPase activity of normal *ras* was stimulated by NF-1 GRD, while two different activated *ras* mutant proteins were unaffected (Martin et al. 1990; Ballester et al. 1990; Xu et al. 1990). The affinity of NF-1 GRD for $p21^{ras}$ was 20 times higher than that of rasGAP, suggesting that NF-1 is a potential regulator of $p21^{ras}$ activity, especially when cellular $p21^{ras}$ concentrations are low (Martin et al. 1990).

Further evidence for a role of *NF-1* as a regulator of *ras* genes was derived from complementation experiments in yeast. *IRA1* and *IRA2* genes of *S. cerevisiae* function upstream of *RAS1* and *RAS2* genes. Their products downregulate *RAS* activity by stimulating the GTPase activity of *RAS*. *ira*$^-$ mutants are characterized by sensitivity toward heat shock and lack of glycogen accumulation (Tanaka et al. 1989, 1990a,b, 1991). Similar phenotypes can be detected in yeast cells expressing mutated *RAS2* genes. Expression of NF-1 GRD cloned in a yeast expression vector was shown to suppress the heat shock sensitive phenotype of *ira*$^-$ mutants. Moreover, the ability to store glycogen was restored by NF-1 GRD (Xu et al. 1990; Martin et al. 1990; Ballester et al. 1990). Thus, NF-1 GRD functions similarly to mammalian GAP in the complementation of yeast mutant defects. The biochemical similarity of the rasGAP, NF-1 and IRA2 proteins is also

reflected in their sensitivity toward inhibition of GTPase-activation by arachidonic acid (Golubic et al. 1991, 1992). The product of the *NF-1* gene does not contain SH2 or SH3 domains (Marchuk et al. 1992). Therefore, it is unlikely to be regulated through the same tyrosine kinases that are involved in the control of $p120^{GAP}$.

Antisera were raised to the bacterially expressed GAP-related domain (DeClue et al. 1991; Hattori et al. 1992), to the C-terminus (Hattori et al. 1992), and to the the regions of the *NF-1* gene product that flank the GRD (Daston et al. 1992). The size of the protein detected by these various antibodies was estimated between 220 and 320 kDa (DeClue et al. 1991, Hattori et al. 1992; Daston et al. 1992; Golubic et al. 1992; The et al. 1993). The protein was detectable in the particulate subcellular fraction in brain, but not in spleen, thymus, kidney, liver, lung, and heart (Hattori et al. 1992). Golubic et al. (1992) also described abundant expression in the brain; however, most of immunoprecipitated neurofibromin was present in the soluble rather than the particulate fraction. Immunostaining of tissue sections from the nervous system indicated that neurons, oligodendrocytes, and nonmyelinating Schwann's cells express the *NF-1* gene product (also named neurofibromin), while astrocytes and myelinating Schwann cells do not express detectable amounts (Daston et al. 1992).

Two alternatively spliced exons have been described for the human *NF-1* gene (Xu et al. 1990; Marchuk et al. 1991). When cDNA sequences of the NF-1 GRD were examined from several brain tumor cell lines, a novel form of NF-1 GRD cDNA was isolated. This cDNA contains an extra 63 bases encoding 21 amino acids inserted into the region of the GAP-related domain (Nishi et al. 1991; Suzuki et al. 1991). Type I transcripts without the insertion were found to be predominantly expressed in undifferentiated cells such as fetal brain, while type II transcripts containing the 63-bp insertion were detected in differentiated tissue and in most primary brain tumors analyzed (Teinturier et al. 1992). On treatment with the differentiation-inducer retinoic acid (RA), the predominant NF-1 GRD expression changed from type I to type II in human SH-SY5Y neuroblastoma cells. An astroytoma cell line that does not respond to RA showed no notable change in NF-1 GRD isoform expression (Nishi et al. 1991). It is not known how the catalytic properties of the protein are changed by the amino acid insertion; however, analysis of the predicted secondary structure indicated that the activity may be modified (Nishi et al. 1991; Suzuki et al. 1991). Although the predicted additional NF-1 peptide exhibits 42% amino acid identity with nucleoside triphosphatase I, its functional significance is not known (Teinturier et al. 1992). Recently it was shown that both forms of NF-1 GRD were able to complement loss of *IRA* function when expressed in yeast (Andersen et al. 1993). Another NF-1 cDNA encoding a truncated protein was de-

scribed recently that might also be involved in the regulation of activity in cellular signal transduction. The predicted protein of 551 amino acids lacks the GAP/IRA-like region, but shares the NH_2-terminal 547 amino acid residues with authentic NF-1 protein (Suzuki et al. 1992).

$p21^{ras}$ can be regulated by rasGAP and NF-1 (neurofibromin) GAP in the same cell. Although a significant proportion of the NF-1 protein was found in monomeric configuration in the P100 membrane fraction of cells, it is associated with other cellular proteins to form complexes of 600–800 kDa as shown by glycerol gradient centrifugation after gentle cell lysis (DeClue et al. 1991). In contrast, the GAP protein was found mainly in the cytoplasm (Trahey and McCormick 1987) and may translocate to the plasma membrane upon phosphorylation on tyrosine residues (Ellis et al. 1990; Molloy et al. 1989). This difference between NF-1 and GAP proteins with respect to subcellular localization has important implications for the regulation of the target molecule $p21^{ras}$. Following tyrosine kinase receptor stimulation or transformation, Ras may be regulated in an inducible manner by the GAP protein able to shuttle between cytosol and plasma membrane. Alternatively, Ras may be controlled constitutively by constantly membrane-associated NF-1 protein (DeClue et al. 1991). Bollag and McCormick (1991) have compared GAP activities on normal, activated and effector mutants of $p21^{ras}$ by measuring the intrinsic GTPase activities, the binding affinities to ras-GAP and NF-1 GAP, and the stimulated GTPase activities. They found that both GAP proteins bind oncogenic *ras* proteins, but do not stimulate their GTPase activity. Moreover, NF-1 GAP binds to p21 *ras* up to 300 times more efficiently than rasGAP. In extracts of mammalian cells, both GAP activities are present. GAP activities can be selectively inhibited by certain lipids that play a role as second messengers in signal transduction. Furthermore, the detergent *n*-dodecyl-β-D-maltoside specifically inhibits NF-1 GAP. GAP activity in brain cell extracts was described that was not inhibited by anti-NF-1 and anti-GAP antibodies, suggesting the presence of yet another protein with GAP activity (Hattori et al. 1992). The neurofibromin-GRD peptide exhibited a 20-fold higher affinity for $p21^{Ha\text{-}ras}$ than GAP (Martin et al. 1990). Apart from the differences in the stimulation of the intrinsic GTPase activity of Ras, NF-1 GAP (neurofibromin) and rasGAP can be distinguished by the nature of their physical interaction with the proto-oncogene product (DiBattiste et al. 1993).

6.4.2 Mutations in Human Tumors and Abnormal Regulation of $p21^{ras}$

Malignant schwannoma cell lines derived from tumors removed from patients diagnosed with NF-1 expressed low to almost undetectable levels of NF-1 protein. The cell lines expressed normal levels of $p120^{GAP}$ and $p21^{ras}$.

In spite of a high GTPase activity indicative of normal *ras* gene function and of normal $p120^{GAP}$ levels, these cell lines contain about 50% of Ras in the GTP-bound (i.e., activated) state which represents a tenfold elevation when compared to the level of Ras-GTP in normal cells (DeClue et al. 1992; Basu et al. 1992). A cDNA construct containing the catalytic domain of GAP linked to the *neo* gene was introduced by transfection into the ST88-14 schwannoma line that expressed very little NF-1 protein. Many of the transfected cell clones showed a flat, nonrefractile morphology. Some of the revertants tested grew poorly in semisolid agar medium and exhibited a reduced ability to form colonies. The level of GTP bound to Ras was reduced to that of normal control cells. Thus, the reversion of ST88-14 cells was most likely a direct consequence of the reduction in Ras-GTP due to expression of the GAP catalytic protein domain (DeClue et al. 1992). Since Ras confers a positive proliferative signal on these cells, NF-1 GRD appeared to be a major negative growth regulator in those neural crest-derived cells (Basu et al. 1992). These results demonstrated that an aberrant Ras signaling cascade is an important mediator of malignancy in neural crest derived tumors that are devoid of *ras* mutations (Bos 1989; for review see also Bollag and McCormick 1992).

Li et al. (1992) identified a somatically acquired point mutation affecting codon 1423 of the *NF-1* gene in one single sample from each of 22 colon adenocarcinomas, 10 anaplastic astrocytomas, and 28 peripheral blood samples from patients with myelodysplastic syndrome. The mutations altered a lysine residue in the catalytic domain of neurofibromin which is invariant among GAP-related proteins. A mutation in the same codon was identified in a family with neurofibromatosis type 1. To assay the functional consequences of these mutations, genetically engineered NF-1 cDNA encoding the GRD was expressed in a *Baculovirus* transfer vector. The mutant NF-1 proteins harboring either a Glu or a Gln residue instead of a Lys residue were purified from Sf9 insect cells and their GTPase-stimulating activity assayed. The activities of the Lys to Gln and Lys to Glu mutants were reduced by approximately 400- and 200-fold, respectively, while the affinities of either N-ras-GTP or Ha-ras-GTP for mutant and wild-type NF-1 GRD remained alike (Li et al. 1992). Legius et al. (1993) described the homozygous inactivation of the *NF-1* gene in a fibrosarcoma from a patient with neurofibromatosis type 1. Loss of heterozygosity was found for all chromosome 17 polymorphisms analyzed. The remaining chromosome 17 had a 200-kb deletion of the *NF-1* gene. Further evidence for NF-1 gene mutations in tumors not commonly found in neurofibromatosis type I patients was provided by analysis of neuroblastoma cell lines. Four out of ten cell lines expressed little or no neurofibromin, two of those showed abberrant restriction fragments within the *NF-1* gene (The et al. 1993). Interes-

tingly, NF-1 deficient neuroblastoma cell lines showed high levels of Ras-GTP. Their proliferation could be inhibited by overexpression of $p120^{GAP}$. The authors speculated that NF-1 is required in a *ras*-mediated differentiation pathway which is blocked by mutations in tumor cells. A homozygous deletion of most of the NF-1 was detected in one out of eight melanoma cell lines analyzed. NF-1 cDNA and protein were not detectable in this cell line (Andersen et al. 1993).

Overexpression of NF-1 GRD cDNA encoding amino acid residues 1194–1531 in v-Ha-*ras* transformed NIH/3T3 fibroblasts significantly reduced their ability to proliferate without anchorage. With the help of a series of truncated NF-1 GRD constructs the region of the protein required for stimulation of c-Ha-*ras* GTPase was further narrowed down. A subdomain of 91 amino acids ranging from residue 1441–1531 activated the c-Ha-*ras* GTPase as shown by nucleoside release assays. It is not yet known whether these amino acids are sufficient to confer the anchorage-dependent phenotype on *ras* transformed cells (Nur-E-Kamal et al. 1993). As indicated by Downward (1992), it not yet formally proven that the full-length NF-1 protein has GAP activity towards $p21^{ras}$.

6.5 Ha-*ras*

The transfection of plasmid DNA containing both the nonmutated Ha-*ras* proto-oncogene and the mutant Ha-*ras* gene from T24 bladder carcinoma cells (Reddy et al. 1982) into immortalized rat 208F cells resulted only in a low frequency of transformed colonies. Most of the colonies were morphologically normal, anchorage-dependent, and nontumorigenic. The normal phenotype was induced even in stable mutant *ras* transfectants, into which the normal *ras* gene was introduced subsequently. Suppressed colonies expressed more normal than mutant $p21^{ras}$ (Spandidos and Wilkie 1988). Introduction of the normal Ha-*ras* gene under the control of its own promoter had only little effects on the tumorigenic phenotype of the human bladder carcinoma cell line T24. When the endogenous Ha-*ras* promoter was replaced by a metallothioneine promoter, a marked, but unstable suppression of the transformed phenotype was observed. The authors suggest that the suppression is caused by competition of the normal Ha-*ras* gene product with the mutant protein for cellular targets, for example, the GAP protein. It is certainly interesting to note that cancer cell lines often express only mutant alleles of *ras* genes (Feinberg et al. 1983; Santos et al. 1984; Capon et al. 1983). Loss of the normal *ras* allele and retention of a mutated allele is also frequently found in primary human tumors (for review see Bos 1989). There are cell lines and tumors that coexpress a mutant and a normal

ras allele (Shen et al. 1987; Paterson et al. 1987, Bos 1989). It is interesting to note that the replacement of one normal allele of the Ha-*ras* gene by a mutated copy did not result in neoplastic transformation of immortalized Rat1 cells provided that the mutant allele was not overexpressed (Finney and Bishop 1993). The results reported by Spandidos and Wilkie (1988) are in contrast to the finding that overexpression of the normal *ras* proto-oncogene results in neoplastic transformation of immortalized mouse NIH/3T3 cells (Chang et al. 1992). The reason for the differential sensitivity of cell lines toward introduction of the proto-oncogene is not understood. Possibly, sensitive cells such as NIH/3T3 have sustained alterations in critical regulatory circuits resulting in uncontrolled proliferation due to an enhanced Ras-GTP pool. Mutations in upstream or downstream effectors may stimulate mitogenic signaling in these cells. Preneoplastic NIH/3T3 cells and rat 208F cells differ in their ability to resist *ras* oncogene-mediated transformation. Cell fusion studies revealed that rat 208F cells were able to suppress the neoplastic phenotype when hybridized to Ha-*ras*-transformed rat FE-8 cells. In contrast, mouse NIH/3T3 cells had lost suppressive activity (Iten et al. 1989).

6.6 *Thy*-1

Sugimoto et al. (1991) have examined the levels of several cell surface proteins in oncogene-transformed NIH/3T3 fibroblasts using fluorescein isothiocyanate (FITC) conjugated antibodies and a fluorescence-activated cell sorter. The proteins included fibronectin, collagen type IV, laminin, Thy-1, and the histocompatibility antigen H2 K. The levels of Thy-1 protein and its mRNA were significantly reduced in DT cells and cell line 635, two derivatives of NIH/3T3 containing two or one copy of KiMSV, respectively. Thy-1 is a cell surface glycoprotein anchored to the cell membrane by glycophosphatidyl-inositol (Low et al. 1985; Tse et al. 1985). The function of Thy-1 is still unknown. It has been implicated to play a role in cell-cell recognition (Williams 1985), particularly in development (Morris 1985; Stern 1973), as well as in proliferation and differentiation of different cell types (Chen et al. 1987; Kollias et al. 1987). The level of Thy-1 mRNA in untransformed NIH/3T3 and *ras* transformed derivatives is probably regulated by a degradative pathway rather than by its synthesis as shown by nuclear runoff transcription assays. Since there was no absolute correlation between Thy-1 downregulation and expression of malignant properties in *ras* transformed cells and phenotypic revertants (Noda et al. 1983; 1989), Sugimoto et al. determined the effect of Thy-1 overexpression on the susceptibility of NIH/3T3 cells toward transformation by KiMSV. Following KiMSV-infection, Thy-1 negative NIH/3T3 cells produced 3-times as many

colonies in semisolid agar medium as Thy-1-positive cells. Transfection of *Thy-1* cDNA into *ras*-transformed NIH/3T3 resulted in the partial inhibition of anchorage-independent growth. In addition, *Thy-1* overexpression in *ras* transformed A1 cells resulted in lower colony-forming efficiency in soft agar and reduced tumor formation in nude mice. In contrast, DT cells did not allow a high *Thy-1* expression after transfection of *Thy-1* cDNA and continued to express the transformed phenotype.

6.7 *gas*-1

The expression of a number of genes is increased upon serum starvation or density inhibition of normal fibroblasts. Several growth arrest-specific genes (*gas* genes) were isolated by differential cDNA hybridization (Schneider et al. 1988; Manfioletti et al. 1990). The product of the *gas*-1 gene is an integral membrane protein of 384 amino acids containing two putative transmembrane domains flanking an extracellular region encompassing amino acids 75–363 (Del Sal et al. 1992). When *gas*-1 was overexpressed from a constitutive promoter in quiescent cells, the serum-induced transition from the G_0 to the S phase of the cell cycle is blocked. However, the ectopic expression of *gas*-1 does not inhibit induction of immediate early proto-oncogenes *fos* and *jun*. Normal cycling cells were able to respond to an increased expression of *gas*-1 by exiting from the cell cycle. Ectopic expression of *gas*-1 driven by an SV40 promoter following microinjection of plasmid DNA resulted in a significant inhibition of DNA synthesis in NIH/3T3 cell lines transformed by either v-*ras*, v-*src*, v-*fos*, or v-*myc* oncogenes. SV40 transformed NIH/3T3 cells were insensitive toward *gas*-1 inhibition. Reduced expression of *gas*-1 was reported in Ki-*ras* transformed NIH/3T3 cells and primary mouse fibroblasts (Cairo et al. 1992). However, high levels of *gas*-1 mRNA were detected in rapidly proliferating fibrosarcoma and rhabdomyosarcoma maintained as xenografts on syngeneic mice. Therefore, the authors concluded that *gas*-1 expression was insufficient to inhibit proliferation and revert the tumorigenic phenotype of these cells.

The *gas*-2 gene encodes a protein of an apparent molecular mass of 36 kDa that is a component of the microfilament system. The *gas*-2 protein localizes with actin fibers, at the cell boundary and along stress fibers in growth-arrested NIH/3T3 fibroblasts. While *gas*-2 expression increased in serum-starved nontransformed cells, expression did not increase in cells transformed by v-*ras*, v-*src*, v-*fos*, and v-*myc* oncogenes (Brancolini et al. 1992). The effect of *gas*-2 overexpression in oncogene-transformed cells after introduction of the gene has not been reported.

6.8 *p53*

Molecular analysis of human cancers has recently provided convincing evidence for the multistep process of tumorigenesis that was initially suggested by Foulds (1958). Particularly, the genetic lesions occurring in colorectal tumors have been analyzed in much detail. As summarized by Fearon and Vogelstein (1990), the accumulation of at least four or five genetic alterations is thought to be required for the initiation of neoplasia and the gradual progression of normal colonic epithelial cells into a malignant tumor cell population. These alterations include the mutational activation of proto-oncogenes, especially of Ki-*ras*, and the functional inactivation of tumor suppressor genes such as *p53, MCC/APC,* and *DCC*. Similar combinations of genetic changes may occur in other types of human tumors as well. Many human tumors have been screened for mutations in the *p53* tumor suppressor gene and in *ras* oncogenes (for review see Levine et al. 1991; Hollstein et al. 1991; Bos 1989). To date, mutation of *p53* is regarded as the most frequently found genetic alteration in human tumors. The p53 phosphoprotein was originally detected as a tumor antigen of 393 amino acids in cells transformed by SV40. The protein was shown to form physical complexes with the large T antigen of SV40 and with the oncogene products of other DNA tumor viruses. The p53 protein can bind to double-stranded DNA as well as to RNA and can act as a regulator of transcription. It can stimulate the transcription of some genes such as the muscle creatine kinase gene, but also repress the transcription of other genes such as the c-*myc* oncogene. Mutations in the *p53* gene that are clustered into hot spots at least in some tumors result in an increase of the half-life of its protein product and accumulation in tumor cells. However, the normal wild-type protein may be accumulated also in normal cells after exposure to DNA damaging agents such as irradiation or drugs (for review see Lane 1992).

Mutations in the *p53* or one of the different *ras* genes can exist in the same cell, consequently they provide an interesting model to study the functional consequences of suppressor gene inactivation and oncogene activation. Mutant and wild-type (wt) *p53* genes exerted fundamentally different activities in cotransfection experiments with activated *ras* genes. Some, but not all mutated *p53* genes were able to act as immortalizing oncogenes in cotransformation experiments using rat embryo fibroblasts as recipients (Levine et al. 1991; Michalovitz et al. 1991; Farrell et al. 1991). Moreover, the introduction of mutant *p53* genes into tumorigenic cells that had lost both wild-type alleles resulted in an enhacement of malignant properties (Michalovitz et al. 1991; Zambetti et al. 1992; Gerwin et al. 1992). However, wild-type *p53* genes appeared to inhibit the expression of neoplastic transformation.

6.8.1 Negative Growth Regulation by p53 in the Presence of Activated ras Oncogenes

The p53 expression plasmids initially used for transfection assays contained mutations and thus did not resemble the true wild-type gene. Wild-type p53 expression plasmids were shown to inhibit the formation of transformed foci derived from normal rodent fibroblasts when introduced simultaneously with cooperating oncogenes such as *myc* plus *ras* and adenovirus E1A plus *ras* (Eliyahu et al. 1989; Finlay et al. 1989). Clones displaying a transformed morphology were infrequent in these transfections. Although all of those contained the *p53* gene integrated in their genome, these clones either failed to express the protein or expressed a mutated form of it (Finlay et al. 1989). In contrast to normal embryo fibroblasts, an established rat cell line, Rat-1, was only marginally sensitive toward inhibition by the normal *p53* gene. Cotransfection of *ras* or *src* oncogenes together with wt *p53* also did not result in a significant reduction in the number of transformants (Eliyahu et al. 1989).

The growth-inhibitory activity of wt *p53* was also investigated in human carcinoma cell lines. Baker et al. (1990) have introduced wt *p53* into SW 480 colon carcinoma cell line harboring an activated Ki-*ras* oncogene. Colony formation of wt *p53* transfectants was reduced five-to tenfold as compared to mutant *p53* transfectants. Colonies obtained after wt *p53* transfection contained rearranged or deleted exogenous *p53* DNA sequences and *p53* mRNA expression was not detectable. The carcinoma cells expressing the wt *p53* gene were unable to progress through the cell cycle. The growth inhibition of wt *p53* was not due to unspecific toxicity of its protein product, since the growth of epithelial cells derived from a benign colorectal tumor (VACO 235) was unaffected (Baker et al. 1990). The antiproliferative activity of a gene on chromosome 17 was confirmed by another group in an indirect way. Goyette et al. (1992) introduced chromosomes 5, 15, 17, or 18 into SW 480 colon carcinoma cells via microcell fusion. Microcell hybrids containing chromosome 18 produced slowly growing tumors in only some of the animals injected, whereas chromosome 5 hybrids were strongly suppressed for tumorigenicity and exhibited morphological alterations. Transfer of chromosome 17 into SW 480 cells did not yield viable colonies suggesting that this chromosome carries an active growth suppressor. Transfer of chromosome 15, which was regarded as a chromosome irrelevant for cell growth control, did not induce any phenotypic changes in SW 480 target cells. Sharma et al. (1993) compared the antiproliferative effect of wt *p53* on human EJ bladder carcinoma, SW 480 colon carcinoma, and HT 1080 fibrosarcoma cell lines harboring activated Ha-*ras*, Ki-*ras*, and N-*ras* oncogenes, respectively. As shown by both transient and stable transfections, wt *p53*

was antiproliferative irrespective of the status of the endogenous *p53* gene. These studies supported the notion that wt *p53* exerts a strong antiproliferative effect on tumor cells expressing Ki-*ras*.

The simultaneous effect of wt *p53* and oncogenes on the proliferation of murine NIH/3T3 cells was investigated by Chen and Defendi (1992). Expression of exogenous wt *p53* resulted in a more than 95% reduction of colony formation. This antiproliferative effect was partially abolished by cotransfection of activated human Ha-*ras*, since the yield of colonies inreased about tenfold. The wild-type *p53* gene had no effect on in vitro transformation in *ras*-transformed NIH/3T3 cells. Transfected cells continued to grow in low serum medium, to exhibit a transformed morphology and anchorage independence. In contrast, HPV18 E6 and c-*myc* oncogenes were able to overcome the antiproliferative but not the antitransforming effects of wt *p53*. The functional interaction of *ras* oncogenes and endogenous wt *p53* genes was investigated in reconstituted mouse prostate organs (Lu et al. 1992). Epithelial and mesenchymal cells were isolated from fetal mouse urogenital sinus and infected with recombinant retroviruses carrying marker genes and also *ras* or *myc* oncogenes. The manipulated cells were grafted into the ventral kidney capsule of mice, where they differentiated into prostatelike tissue (Thompson et al. 1989). In reconstituted mouse prostate, the *ras* oncogene caused hyperplastic growth (R cells), whereas the combinations of *ras* and *myc* genes (RM cells) resulted in the formation of carcinomas. Mutations in the endogenous *p53* gene were not found in these tumors. In RM cells, the expression of endogenous wt p53 was high, but very heterogenous. Cell cycle control and mitosis were perturbed, as indicated by the presence of micronuclei, giant and multinucleated cells. RM cells were completely unresponsive toward the growth-inhibitory activity of exogenous wt p53. Instead, R cells harbored *p53* mutations and were growth-arrested by exogenous wt *p53*. Thus, the susceptibility of *ras*-expressing cells toward wt *p53* is governed by the state of endogenous *p53*. The defective growth control function was restored by introduction of wt *p53* into R cells. The coexpression of *myc* bypassed the need for *p53* mutations in RM cells. Introduction of wt *p53* no longer had an antiproliferative effect.

7 Discussion and Perspectives

The reversion of *ras*-mediated transformation can be achieved by different means such as treatment with certain antibiotics and cytokines, expression of antisense RNA or oligonucleotides to the oncogene, or the forced expression of transformation suppressor genes. Most importantly, *ras* oncogene-ex-

pressing cells may loose their oncogenic properties in vivo and in vitro even if the oncoprotein level is unaltered. The expression of the neoplastic phenotype may be blocked by inactivation of an effector molecule (or molecules) essential for the transduction of the mitogenic signal such as in effector$^-$ mutants or by the activation of an endogenous suppressor gene acting in a dominant manner. Cell lines that are resistant toward *ras* transforming activity may have sustained similar alterations as revertants obtained from transformed cells. Several genes have been identified that are directly or indirectly associated with the suppression of neoplastic transformation. From the study of those putative or proven growth suppressor genes, the following mechanisms of suppression have emerged: the expression of the *ras*-induced transformed phenotype may be perturbed at the level of the signal transduction cascade downstream of Ras, at the level of transcriptional control, and possibly at the level of cellular interactions with the extracellular matrix and with neighboring cells. Two genes, *Krev*-1 and *rsp*-1, were identified by expression cloning, the method that provides the most direct approach to molecular clones of transformation suppressor genes. However, both genes exerted their suppressing activity only when overexpressed in *ras*-transformed cells. This raises the question, whether expression of these genes at physiological levels would also confer any constraints on cellular growth. An important hint for a role as regulators of normal proliferation or differentiation would be the detection of inactivating mutations in tumorigenic cells. Mutations in the *rsp*-1 gene have not been reported and the search for alterations of *Krev*-1 in experimental and human tumors has been rather discouraging. In contrast, the *NF-1* gene encoding neurofibromin was isolated by positional cloning based on its association with von Recklinghausen neurofibromatosis type 1. Mutations or deletions were found in NF-1-associated tumors and in a few cell lines derived from other tumors. It is intriguing that neural crest derived tumors found in neurofibromatosis type1 patients have not sustained oncogenic mutations in *ras* genes. However, loss of neurofibromin expression abolishes GAP activity and causes an elevation of activated Ras-GTP. Thus, a continuous mitogenic signal can be generated, although the GTPase activity of Ras itself is not affected by mutations. Similarly, pertubation of rasGAP activity could result in Ras activation as suggested by the inhibition of *ras*- or *src*-induced transfomation by overexpression of *ras* GAP. Unlike the *p53* tumor suppressor gene, the genes encoding rasGAP or neurofibromin (NF-1 GAP) represent a large target for mutations, due to their considerable size. For the same reason, both genes were unlikely to be detected by gene transfer and expression cloning. The important function of these proteins in Ras regulation warrants further screenings for mutations in tumors.

Several genes were specifically expressed in phenotypic revertants derived from *ras*-transformed cells. Reversible downregulation of gene expression during the conversion from the normal to the transformed state and reexpression upon reversion, or selective expression in revertants does not define a causal role in those phenotypic changes. Genes encoding different types of collagens or other components of the extracellular matrix were preferentially detected by differential hybridization techniques, since the corresponding transcripts are quite abundant in revertant cells and may represent the "tip of the iceberg." It is very unlikely that these genes were associated with the revertant state by coincidence, since they were repeatedly identified in independent sets of revertants. These genes, coined class II suppressor genes, may serve as important markers for the normal and the revertant state (Lee et al. 1991). It is interesting to note that the genes encoding collagens and lysyl oxidase (*rrg*) appear to be coordinately downregulated on *ras* transformation. Reexpression of some structural components of the extracellular matrix and of the enzyme lysyl oxidase mediating collagen and elastin cross-linking may have, at least indirect, growth-constraining effects on cells. Cellular proliferation and proto-oncogene activity can be modulated by cell shape (Folkman and Moscona 1978; Tucker et al. 1981; Kulesh and Greene 1986; Farrell and Greene 1992). This notion is supported by the finding that overexpression of the fibronectin receptor (Giancotti et al. 1990) and of vinculin (Fernandez et al. 1992) in spontaneously transformed cells restores normal growth control.

The *p53* gene serves as a paradigm for class I suppressor genes (Lee et al. 1991), since its expression in tumorigenic cells had profound inhibitory effects on cellular proliferation and on transformed phenotypes. P53 is an important transcriptional activator which probably regulates a number of genes associated with cell survival, proliferation and differentiation. Due to its predominantly nuclear localization, the p53 protein does not directly interfere with elements of the Ras signaling pathway. It may, however, interfere with several downstream targets of Ras. Thus, *p53* expression may result in growth arrest even in the presence of Ras, particularly as long as the level of expression is very high. The existence of different signaling pathways leading to growth stimulation would explain why some *ras*-expressing cells failed to respond to growth inhibition by p53. Alternatively, these cells may have sustained mutations in the p53 pathway.

The multistep process of malignant transformation is tightly associated with the inactivation of suppressor gene activity. On the basis of chromosome transfer studies, it was proposed that restoration of a single missing suppressor activity is sufficient to cause phenotypic reversion, even if several other lesions including *ras* mutations persist (Goyette et al. 1992). If this concept is validated by direct analysis of the genes involved, successful

therapeutic intervention would require restoration of single defects only. The restoration of a mutated or deleted gene function requires reintroduction of the wild-type gene into cancer cells in vivo, an approach which might be feasible in the not too distant future. An alternative view is that the restoration of normal growth control requires the coordinate action of several genes. Since the reverted phenotype is inducible by certain drugs and antibiotics, it is tempting to speculate that the genetic program underlying the expression of the normal state can be reactivated pharmacologically.

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Note added in proof

A number of recently published papers describing new revertant cell lines, genes associated with *ras* reversion, and inhibitors of *ras* expression or function could no longer be discussed in this article. Of particular interest is the finding that several genes are able to suppress a variety of the parameters of neoplastic transformation mediated by *ras*. These genes include a deletion mutant of the proto-oncogene c-*jun* (Brown et al. 1993) and the genes encoding the cognate heat shock protein hsc 70 (Yehiely and Oren 1992), tropomyosin-1 (Prasad et al. 1993) and the mutant form of gelsolin (Müllauer et al. 1993).

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Regulation of Gene Expression by Prolactin

W. DOPPLER

Contents

1 Introduction: Prolactin – A Multifunctional Regulator

Long before the polypeptide hormone prolactin was biochemically characterized, the existence of a lactogenic hormone was postulated. Extracts from the anterior lobe of the pituitary stimulated the mammary gland to synthesize milk protein genes (Stricker and Grueter 1928). Later on, a great number of

Institut für Medizinische Chemie und Biochemie der Universität Innsbruck, Fritz-Pregl-Strasse 3, A-6020 Innsbruck, Austria

other physiological effects were reported to be triggered by the hormone. When Nicoll (1974) wrote his comprehensive review on the physiological actions of prolactin, the number of reported actions of prolactin already exceeded the number reported for all other adenophysial hormones combined. They include effects on the growth and differentiation of organs involved in reproduction, namely, ovary, testis (Dombrowicz et al. 1992), prostate (Pérez-Villamil et al. 1992), and mammary gland. Prolactin was also found to have an effect on the islets of the pancreas (Brelje et al. 1993) and has been described to be involved in osmoregulation (Leontic and Tyson 1977), in the initiation of behavioral effects and in the regulation of the immune system in mammals and birds (Gala 1991; Skwarlo-Sonta 1992). In development, it exerts a juvenilizing action in amphibian metamorphosis by counteracting the effects of thyroid hormone (Tata et al. 1991).

In the vertebrate pituitary, prolactin is synthesized in specialized cells, the lactotrophs. From the five cell types in the anterior pituitary, the lactotrophs appear last near birth in the developing gland. Estrogens induce the expansion of lactotrophs and stimulate the expression of the prolactin gene within these cells. Work on the ontogeny of this cell type has been reviewed recently (Voss and Rosenfeld 1992). Secretion of prolactin from the gland is subject to hypothalamic control and inhibited by dopamine. The secretion is pulsatile with a sexually dimorphic pattern (Arey et al. 1989). This might be important for a differential effect of the hormone on the gene expression in males vs. females as it has been described for the related growth hormone (Mode et al. 1989; Legraverend et al. 1992).

Several reports indicate that prolactin is also produced outside the pituitary in placenta (Hiroka et al. 1991), decidua (Di Mattia et al. 1990) and myometrium (Gellerson et al. 1991). The extra-pituitary produced prolactin encodes for the same size of protein synthesized in lactotrophs. However, the transcripts contain an additional 5'-noncoding exon. Prolactin-specific transcripts and immunoreactive protein were also found in human thymocytes and lymphoid cell lines (O'Neal et al. 1992; Pellegrini et al. 1992). The detection of prolactin transcripts in lymphocytes support the hypothesis that the hormone has also the properties of a cytokine. Since both placenta and thymocytes have been shown to express also prolactin receptors (PLR-R; Sect. 2.1), prolactin can act in an autocrine or paracrine manner there. Two reports describe the expression of prolactin transcripts in the mammary gland of rats (Steinmetz et al. 1993; Kurtz et al. 1993), suggesting that some of the prolactin secreted into the milk is produced directly in the mammary gland.

The hormone is subject to posttranslational modifications. Four differently glycosylated forms of prolactin have been described (Sinha et al. 1991). In the human pituitary, 10%–15% of prolactin is glycosylated. This modification appears to alter the biological activity of the hormone (Sinha et

al. 1991; Markoff et al. 1988). A 16-kDa fragment of prolactin, but not the unprocessed prolactin has been found to specifically inhibit fibroblast growth factor stimulated growth of capillary endothelial cells (Ferrara et al. 1991). A receptor distinct from the PRL-R is postulated to mediate this response (Clapp and Weiner 1992).

In addition to prolactin variants, hormones have been identified which are structurally and functionally related to prolactin but are expressed from different genes. They form the prolactin family of hormones, which include placental lactogens, prolactin-like proteins, and proliferins (Soares et al. 1991). Placental trophoblasts are the major source of these mainly glycosylated peptides. There is a strong species difference in the expression pattern and structure of the various members of this family. Members of the proliferin family have been found only in mice to date. Placenta lactogen found in humans is closely related to growth hormone, whereas the placental lactogens found in nonprimate mammals are more related to prolactin than to growth hormone. In primates, growth hormone can also induce prolactin-like actions. This has been attributed to the unique property of primate growth hormone to bind to the PRL-R. Zinc ion, in complex with the human growth hormone has been described to mediate the high-affinity binding (Cunningham et al. 1990). A more than 1000-fold higher affinity of human growth hormone to a bacterial expressed extracellular domain of the human PRL-R was observed in the presence of zinc ions. In a later report (Rozakis-Adcock and Kelly 1991), high-affinity binding of human growth hormone without prior addition of $ZnCl_2$ was observed. However, the authors could not exclude the possibility that trace amounts of zinc were responsible for the effect. Experiments with transgenic animals harboring the gene for the human growth hormone revealed that human growth hormone is also mammotrophic and lactogenic in mice (Bchini et al. 1991). Again, binding of the human growth hormone to the mouse PRL-R appears to be responsible for that effect. The species-specific evolution of multiple hormones with prolactin-like actions is not surprising in view of the great number of physiological effects induced by prolactin. It is speculated that the diversity increases the fidelity of the complex stage and tissue-specific synthesis, secretion, and delivery of the hormone to the multiple target cells and that some hormones might also have slightly different effects when bound to their receptor (Soares et al. 1991).

In contrast to the abundant knowledge about physiological actions of prolactin and its relatives, progress in the understanding of biochemical mechanisms by which the hormone exerts its multiple specific functions was slow until the end of the last decade. Research was severely impeded by the failure to identify the target molecules of prolactin action in the cell. The situation has changed dramatically since the cloning of the PRL-R from rat

liver in 1988 (Boutin et al. 1988). In addition, the identification of *cis*-acting sequences mediating the response to prolactin (Doppler et al. 1989) in the promoter of milk protein genes has set the stage to identify nuclear factors involved in the regulation of gene expression by prolactin. Subsequent work has established unforeseen and exciting relationships in the mechanisms by which prolactin or other extracellular signaling proteins influence gene expression.

2 The Prolactin Receptor: Member of a Large Family

2.1 Structure and Expression

A wealth of information about the structure of the PRL-Rs expressed in mammals was already available 3 years after cloning of the rat liver receptor. To date, the primary structure of PRL-Rs has been described from five mammalian species (Table 1). The receptors are organized into an extracellular region, a short transmembrane region, and an intracellular region of variable length. When expressed in COS cells, the cloned receptors bound prolactin and human growth hormone with approximately the same affinity ($2–3.10^{-10}$ *M*) previously described for purified receptor preparations. The hormones of the prolactin family also bind with high affinity. There is some evidence for the existence of a distinct receptor for placental lactogen (Freemark and Comer 1989) with distinct binding behavior when compared to the PRL-R. However, the cloning of such a receptor has not been described so far.

In all animal species studied, several transcripts were specifically hybridizing to PRL-R complementary DNA (cDNA)-derived probes (Table 1).

Table 1. Cloned forms of the prolactin receptor

Species	Transcript (kbp)	Protein size (amino acids)	Reference
Human	2.8, 3.5, 7.3, 2.7, 3.5, 8.6, 10.5	598	Boutin et al. 1989 Ormandy et al.1993
Cow	2.6, 3.8, 4.4	557	Scott et al. 1992
Rabbit	2.8, 4, 6.5, 10 2.7, 3.4, 6.2, 10.5	592	Edery et al. 1989 Dusanter-Fourt et al. 1991
Rat	1.8, 2.5, 3, 5.5 1.8, 2.1, 2.6, 4.6, 9.7 6.7, 10.1	291, 591	Shirota et al. 1990; Boutin et al. 1988 Hu and Dufau 1991 Dardenne et al. 1991
Mouse	1.4, 2.4, 3.5, 4.2, 8.3, 9, 10	292, 303, 310, 597	Davis and Linzer 1989a; Clark and Linzer 1993; Buck et al. 1992

They appear to result from differential splicing of one primary transcript (Kelly et al. 1991; Dusanter-Fourt et al. 1991). A final proof for this notion is expected to come from the publication of the exon/intron structure of a PRL-R gene. In humans, cows, and rabbits, the available cDNA sequences suggest that only one form of the PRL-R protein is expressed, with a size ranging from 557 amino acids in cows to 598 amino acids in humans. In rats, two receptor forms – a short form and a long form – were identified. These two receptors have identical extracellular domains but differ by 300 amino acids in the length of their intracellular domain. Mice exhibit the most complex pattern of PRL-R gene expression. Filter hybridization analysis of transcripts has revealed the existence of at least seven transcripts in mouse organs (Buck et al. 1992), coding for four different receptor proteins (Clarke and Linzer 1993; Table 1). Again, these four murine receptor forms have identical extracellular domains and differ only in the cytoplasmic part of the receptor. The prolactin binding proteins found in serum (Amit et al. 1992) and in milk (Postel-Vinay et al. 1991) might also be encoded by some of the PRL-R transcripts, as it has been described for the growth hormone-binding protein (Tiong and Herington 1992 and references therein).

Our present knowledge about the expression pattern of the PRL-R in animals and cell lines is mainly derived from experiments measuring receptor-specific transcripts or from the analysis of the specific binding sites for prolactin. The availability of suitable antibodies has limited so far a direct determination of receptor protein levels and sizes in tissues. Transcripts of the receptor were found in many organs (Kelly et al. 1991). Especially high levels were detected in the female liver, in the ovary, and in the mammary gland. Polymerase chain reaction techniques allowed detection of receptor transcripts in lymphoid tissues, lymphoid cell lines (O'Neal et al. et al. 1991; Pellegrini et al. 1992; Koh and Phillips 1993), and in the hypothalamus (Chiu et al. 1992). The latter results were confirmed by autoradiographic localization of prolactin binding sites (Crumeyrolle-Arias et al. 1993). PRL-Rs were also characterized in the human brain and the choroid plexus (Di Carlo et al. 1992). Monoclonal antibodies directed against the rat PRL-R allowed the identification of PRL-Rs in human and murine thymic epithelial cells (Dardenne et al. 1991). The antibodies were also able to modulate the function of these cells, presumably by activating the receptors.

The significance of the multiple forms of receptor protein expressed in rodents is not clear. The proteins only differ in their intracellular domain, and it has been suggested that this might result in differential coupling to intracellular signaling systems (Davis and Linzer 1989a). Evidence for that notion comes from cotransfection experiments of the long and the short form of the rat receptor with a milk protein gene promoter construct (Lesueur et al. 1991). Only the long form of the receptor was capable of mediating the

effect of prolactin on the expression of the reporter gene linked to the promoter construct. The transcripts for the different receptor forms have been reported to be independently regulated during pregnancy and lactation in a tissue-specific manner (Jahn et al. 1991; Jolicoeur et al. 1989; Hu and Dufau 1991; Buck et al. 1992; Clarke and Linzer 1993). Of special interest is the regulation of receptor transcripts in the ovary during pregnancy: In that organ, prolactin has two well-described functions (Nicoll 1974): It acts as a luteotrophic hormone during pregnancy and thereby stimulates production of progesterone and synthesis of chorionic gonadotropin receptor (Gåfvels et al. 1992) in the corpus luteum. In addition, prolactin promotes as a luteolytic hormone the degeneration of corpora lutea from previous reproductive cycles. It is speculated that the organ serves also in relaying signals between prolactin and members of the prolactin family (Soares et al. 1991). In accordance with this diversity of functional roles, the ovaries are the organs with the most complex pattern of PRL-R transcripts (Hu and Dufau 1991; Buck et al. 1992). In situ hybridizations established a differential expression of the transcripts during pregnancy in granulosa cells and the corpus luteum (Clarke and Linzer 1993). Most interestingly, expression of one short receptor form was uniquely detected in atretic follicles at midgestation, thus implicating a role of this receptor form in mediating the luteolytic functions of prolactin.

The structure of the PRL-R has been compared extensively with published data obtained from other cloned receptors. The closest relative is the receptor for growth hormone (Leung et al. 1987). In addition, the receptors for a number of cytokines including the interferons have regions of relatively low but significant sequence homology with the PRL-R in their extracellular domains (Bazan 1989, 1990; Thoreau et al. 1991; Kelly et al. 1991). On the basis of these findings, the PRL-R has been grouped together with the growth hormone receptor (GH-R) into an expanded cytokine receptor superfamily.

2.2 Mechanism of Activation by Hormone

2.2.1 Site of Interaction with the Hormone

Regions in the extracellular domain of the receptor critically for the binding of the hormone have been mapped (Rozakis-Adcock and Kelly 1991). An N-terminal cystein-rich domain was found to be important for the specificity of binding. In addition, the WS×WS motif (tryptophan, serine, any amino acid, tryptophan, serine) of the receptor, which is conserved in the cytokine superfamily (Bazan 1990), is required for high-affinity binding and it has been proposed that this may serve as a target site for interaction with an

accessory protein necessary for the formation of high-affinity binding sites (Rozakis-Adcock and Kelly 1991). The domains of the hormone required for binding have not been mapped yet. The availability of monoclonal antibodies directed against a number of distinct epitopes of prolactin (Staindl et al. 1987; Scammell et al. 1992) and the analysis of mutated prolactin molecules (Luck et al. 1992) should facilitate the localization. It is usually assumed that the receptor localized in the plasma membrane is contacted and activated by the hormone. There has also been a report on the requirement for nuclear expressed prolactin to stimulate interleukin-2 (IL-2)-dependent proliferation of T lymphocytes (Clevenger et al. 1991), implicating a second site of activation of the PRL-R or another unkown prolactin target in the cell. By contrast, in Nb2 rat lymphoma cells, which are believed to be thymocytes at an intermediate stage of differentiation (Gala 1991), intracellular prolactin had no effect on stimulation of cell proliferation (Davis and Linzer 1988).

2.2.2 *Evidence for Receptor Dimerization*

An important mechanism in the activation of receptors with tyrosine kinase activity is ligand-induced dimerization (Ullrich and Schlessinger 1990). There is now plenty of evidence that the same is true for a number of receptors belonging to the cytokine receptor superfamily (Stahl and Yancopoulos 1993). Cytokine receptors can be classified into four different subgroups by the subunit structure of the formed dimers (Fig. 1). In subgroup A, the ligand binds in a first step with one surface (site 1) to a receptor monomer. In a second step, dimerization is triggered by the contact of a

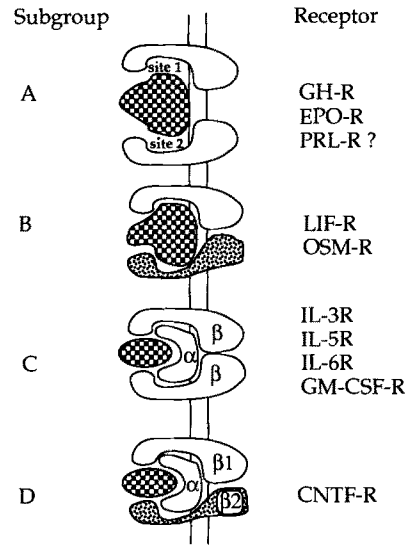

Fig. 1. Subunit structure of dimerized cytokine receptors. In all subgroups one ligand molecule triggers the dimerization of two receptor subunits. In subgroups *C* and *D*, an additional α component of the receptor is involved in the binding of the ligand and in the formation of the dimer. Receptor homodimers (subgroups *A* and *C*) or heterodimers (subgroups *B* and *D*) have been described. In subgroup *A*, the two contact surfaces (*site 1* and *site 2*) of the ligand with the receptors are indicated. For abbreviations see text

second assymetric surface (site 2) of the ligand to a second receptor molecule. The GH-R is the prototype for subgroup A. The crystal structure of two molecules of the GH-R extracellular binding domain complexed by one molecule of growth hormone (Ultsch et al. 1991; de Vos et al. 1992) has been obtained at 2.8-Å resolution, which allowed a precise mapping of the contact surfaces employed by the hormone and receptor molecules (reviewed by Demeyts 1992) and facilitates the rational design of receptor antagonists (Fuh et al. 1993). It is believed that the receptor for erythropoetin (EPO-R) also belongs to subgroup A. Subgroup B comprises the receptors for leukemia inhibitory factor (LIF-R) and oncostatin M (OSM-R). Here, heterodimers of two different receptor subunits, encoded by different genes, are formed by the ligand. In subgroups C and D, dimerization is dependent on an additional receptor subunit (α), which binds specifically to the ligand. Examples are the receptors for the IL-3, IL-5, and IL-6 (IL-3R, IL-5R, and IL-6R) and the granulocyte–macrophage colony-stimulating factor (GM-CSF-R), where the dimerized β-receptor components are homodimers (subgroup C). The ciliary neurotrophic factor receptor (CNTF-R) forms heterodimers of two different components (β1 and β2) in the presence of the cytokine complexed to the α-receptor subunit (subgroup D).

Indirect evidence obtained with Nb2 rat lymphoma cells suggests that the PRL-R belongs to subgroup A (Table 1): First, antibodies were able to activate the receptor presumably by induction of dimerization (Elberg et al. 1990). Second, human growth hormone mutants, with mutations in their binding sites for either the first or second GH-R molecule (first and second site mutants, see also schema for subgroup A receptors in Fig. 1), had functional effects expected for a situation in which PRL-R and the GH-R are dimerizing by the same mechanism: Both site 1 and site 2 mutants showed greatly reduced agonistic effects, whereas only site 2 mutants had antagonistic properties (Fuh et al. 1993). A mutant form of placental lactogen II, which was able to bind to the PRL-R, but was not active in the Nb2 mitogenic assay (Davis and Linzer 1989b), might also fall into the class of site 2 mutants. However, until now there is no direct evidence that the PRL-R can be dimerized by one ligand molecule. Gertler et al. (1993) could only isolate complexes with a 1:1 molar ratio of prolactin and the extracellular domain of the PRL-R. Clearly, further experiments are required for firmly establishing the prevalent mechanism of dimerization.

2.2.3 Intracellular Signaling of the Receptor

What are the signals produced by the activated receptor? With one exception (Zhang et al. 1990), which was not confirmed in a subsequent publication (Nagano and Kelly 1992), inspection of the sequence of the intracellular

domains of the PRL-R did not reveal any sequence motifs that indicate an enzymatic function or a relationship to receptor tyrosine kinases. The same was true for the other receptors of the cytokine superfamily. Very recent work has revealed instead that many, if not all, cytokine receptors have a nonconvalently linked cytoplasmic tyrosine kinase attached to their intracellular domains (Argetsinger et al. 1993). Further work has to establish whether ligand-induced dimerization, followed by activation of attached or intrinsic cytoplasmic tyrosine kinases, is a general principle governing the activation of cytokine receptors and receptor tyrosine kinases. By analogy to the findings with the platelet-derived growth factor (PDGF) receptor tyrosine kinase (Fantl et al. 1992), it is likely that the dimerized PRL-R recruits and activates other signaling proteins in addition to tyrosine kinases. Calcyclin, a cell cycle regulated 10-kDa putative calcium binding protein might be a candidate for such an attached factor (Murphy et al. 1988a). Cross-linking of G proteins to proteins which are immunoprecipitated with antiserum to the PRL-R (Too et al. 1990) suggest that also G-proteins are linked to the PRL-R. The availability of larger quantities of pure PRL-R expressed in insect cells (Cahoreau et al. 1992) should facilitate the identification of proteins with an affinity to the PRL-R by biochemical methods. This will complement functional studies performed with mutated PRL-Rs. An experimental procedure for measuring some of the functional activity of PRL-Rs has already been developed (Leseur et al. 1991; Ali et al. 1992). CHO cells were transiently cotransfected with PRL-R and milk protein gene promoter constructs linked to a chloramphenicol acetyltransferase (CAT) reporter. Measurement of the CAT activity induced by prolactin showed that in this system, the promoter activity was dependend on the form of the cotransfected receptor construct. The long form and the major Nb2 PRL-R form, which is a truncated version of the long receptor form were found to be active, whereas the short form was not functional in this assay.

3 Putative Signaling Pathways Employed by Prolactin for the Regulation of Gene Expression

Effects of the hormone on the tyrosine kinases, protein kinase C (PKC), cyclic nucleotides, intracellular calcium, phospholipases, enzymes regulating the polyamine metabolism and other potential cellular signaling molecules have been investigated extensively. A comprehensive review (Rillema et al. 1988) summarizes earlier work on postreceptor actions. Which of these effects are necessary for the specific expression of prolactin dependent genes and thus play a role in reprogramming the cell after activation of the PRL-R? I will focus here on the evidence for tyrosine kinases, PKC isozymes and

intracellular calcium-mediated signaling being involved in regulating prolactin-dependent gene expression. Since a complete signal transduction cascade intiated by binding of prolactin to its receptor and ultimately effecting the expression of a protein at a transcriptional or posttranscriptional level has not yet been established, our present knowledge about the individual roles of these different signaling systems relies mostly on the results of experiments performed with more or less specific inhibitors.

3.1 Tyrosine Kinases

As discussed in Sect. 2.2.3, one of the first events after binding of the hormone to its receptor is a stimulation of tyrosine kinase activity. The initial observation was made by antiphosphotyrosine immunoblotting experiments. With this technique a rapid phosphorylation of multiple cellular proteins was demonstrated in Nb2 rat lymphoma cells by two independent research groups. A protein designed p120 (Rui et al. 1992) or 121-kDa protein (Rillema et al. 1992) migrated at identical position as a protein induced by growth hormone in 3T3-F442A fibroblasts (Campbell et al. 1993). Subsequent work with specific antibodies (Argetsinger et al. 1993) revealed that this protein corresponds to the ubiquitously expressed tyrosine kinase JAK2 (Wilks et al. 1991). This tyrosine kinase belongs to a family which does not contain SH2 or SH3 domains but harbors the structural features of a second protein kinase activity with unknown function. A new molecular weight of 130 kDa was assigned to that protein in this study. It is not known whether JAK2 is the only tyrosine kinase involved in the signaling of the receptor.

The functional role of JAK2 or other tyrosines on activation of gene expression was evaluated with specific inhibitors. Prolactin-dependent milk protein gene expression was abolished by genistein and staurosporin, but not by lavendustin A (Fan and Rillema 1992; Bayat-Sarmadi and Houdebine 1993). Recently, MGF, a mammary gland specific nuclear factor was identified. DNA binding of this factor is induced directly by prolactin (Sect. 6). The activated factor is recognized by antibodies directed against phosphotyrosine, indicating that it contains phosphotyrosine residues. Genistein and staurosporin but not lavendustin A inhibited the induction (T. Welte, unpublished). Thus, at least one of the effects of tyrosine kinases appears to be mediated by MGF. As discussed later, this nuclear factor is related to the STAT family of transcriptional activators, which comprises factors induced by cytokines and peptide growth factors. It is presently not known, which tyrosine kinase is involved in the phosphorylation of MGF, but JAK2 is obviously a candidate.

In addition to MGF and the JAK2 kinase, other proteins have been observed to be phosphorylated on tyrosine in response to prolactin (Rui et al. 1992; Rillema et al. 1992). It is possible that, upon phosphorylation, these other proteins acquire the capability to bind *src* homology domain 2 (SH2 domain) proteins as it has been demonstrated for the autophosphorylated growth factor receptors (Fantl et al. 1992). Some of these SH2 proteins have been shown to be involved in subsequent steps of cellular signaling. In the case of the receptor for epidermal growth factor (EGF-R) or PDGF-R, SH2 domain proteins link phospholipase C-mediated pathways and signaling regulated by p21 ras to the activation of tyrosine kinases. Such mechanisms might be responsible for the effects of prolactin on PKC and on intracellular calcium.

3.2 Protein Kinase C

PKC is known to comprise a large family of isozymes (Nishizuka 1992) with common structural features. There is no unique activation mechanism for the members of the family. The PKC isozymes α, β, and γ can be activated by calcium and phosholipids, isozymes δ, ε, η and ϑ require phospholipids but are calcium insensitive. There is a third group of atypical isozymes which are not activated by phorbol esters and are insensitive to calcium. The isozymes are differentially blocked in their activity by inhibitors and appear to participate in different signaling pathways in a cell type-specific fashion. So far, investigations published about the role of PKC in prolactin signaling did not take into account the heterogeneity of the PKC family.

Activation of PKC after stimulation of cells with prolactin has been observed in various cellular systems: In mammary gland extracts (Waters and Rillema 1989) and hypothalamic slices (DeVito et al. 1991), prolactin triggered within 15 min the redistribution of PKC from the cytosolic to the particulate fraction of cell extracts. In the mouse mammary cell line NOG-8, where prolactin acts as a mitogen, a similar cellular redistribution is observed already after 5 min (Banerjee and Vonderhaar 1992). Activation of a nuclear PKC has been described in rat liver nuclei (Buckley et al. 1988). The effect of inhibitors of PKC on prolactin dependent cellular functions was analyzed in a number of studies. Whereas two reports document an inibitory effect of H7 (Waters and Rillema 1989; Banerjee and Vonderhaar 1992), another study performed with more inhibitors (Bayat-Sarmadi and Houdebine 1993) led to the conclusion that a protein kinase, which is not PKC, is important in mediating the effect of prolactin. In Nb2 cells, H7 was not effective in blocking the early effects of prolactin on stimulation of DNA synthesis (Rillema et al. 1989).

Attempts to mimic the effect of prolactin by a direct stimulation of PKC with the phorbol ester TPA were only partially successful. In Nb2 cells, TPA increased the mitogenic effect induced by the activation of the PRL-R (Gertler et al. 1985). However, in the same cell line, the phosphorylation pattern of strathmin induced by prolactin is distinct from the phosphorylation induced by TPA (Meyer et al. 1992). These results suggest that the protein kinase important for the stimulation of mitogenesis in Nb2 cells was not one of the PKC isoforms which can be activated by TPA. Consistent results indicating a role of PKC in the prolactin signaling of prostate cells were presented (Franklin et al. 1992): Stimulation of mitochondrial aspartate aminotransferase was observed by addition of TPA and prolactin. Both responses were inhibited by gossypol. Downregulation of PKC by treatment of cells with TPA inhibited the induction. In a number of cell lines, long-term treatment with TPA also resulted in a reduction of PRL-R levels (Ormandy et al. 1993).

A final judgment about the role of PKC in the response to prolactin has to await more refined investigations which account for the heterogeneity in the activation mechanisms and the cell type-specific expression pattern of the isozymes.

3.3 Intracellular Calcium

Intracellular calcium ions modulate the activity of several proteins in the cell. Activation of receptors are often found to induce dynamic temporal and spatial changes in the concentration of this intracellular second messenger. Release of calcium from intracellular stores or calcium influx through specific calcium channels in the plasma membrane temporarily increase the concentration of free calcium in the cytosol. Complex oscillation patterns and calcium waves have been observed with sophisticated techniques (Lechleiter and Clapham 1992). Both frequency and amplitude of such oscillations are believed to be important for the mode of cellular response. Present knowledge about the role of such dynamic calcium responses in the signaling of prolactin is very limited. In Nb2 cells, the role of intracellular calcium ions in mediating the action of prolactin has been studied employing calcium ionophores, $^{45}Ca^{2+}$, calcium channel blockers, and intracellular calcium antagonists (Murphy et al. 1988b). The authors conclude that activation of calcium channels was not an important early event in the mitogenic action of prolactin. Instead, effects obtained with the putative intracellular antagonist TMB-8 pointed to a role of an intracellular calcium pool for mediating the mitogenic effect of prolactin in this cell line. Work on the effect of prolactin on hepatocytes of lactating rats (Villalba et al. 1991) led also to the conclu-

sion that mobilization of calcium from intracellular stores is involved in the induction of glycogen phosphorylase-α activity by prolactin. On both cell types, prolactin acts as a mitogen. Prolactin-induced mobilization of calcium from intracellular stores might be restricted to such cells since in the mouse mammary epithelial cell line HC11, where prolactin induces milk protein gene expression in growth-arrested cells (Doppler et al. 1989), no mobilization of intracellular calcium stores was observed with single-cell imaging techniques. However, a rapid prolactin-induced calcium influx from the extracellular space could be demonstrated (T.Bader and K.Maly, unpublished). A blockade of this calcium influx by ethyleneglycol-bis-β-aminoethylether-N,N,N',N'-tetraacetic acid (EGTA) still allowed the activation of the prolactin-inducible nuclear factor MGF (T.Welte, unpublished), implicating that calcium influx was not required for the activation of this factor. The prolactin-induced increase in intracellular calcium might be important for functions of prolactin other than transcriptional regulation of genes, e.g., regulated exocytosis, which has been shown to be dependent on extracellular calcium in lactating mammary epithelial cells (Turner et al. 1992).

4 Genes Regulated by Prolactin

The number of genes documented to be regulated by prolactin is still small when compared with the great number of physiological actions of the hormone. A summary of such prolactin-induced genes is shown in Table 2. The best studied organ is the mammary gland. For most of the milk proteins (recently reviewed by Rosen et al. 1986, Harris et al. 1990, and Hennighausen et al. 1991) and a number of enzymes involved in the biosynthesis of milk lipids (Barber et al. 1991, 1992a,b) and lactose (Jagoda and Rillema 1991; Peters and Rillema 1992) a positive effect of prolactin on the expression has been demonstrated. This ensures the maintenance of high levels of these proteins during lactation. Lactose synthetase activity requires two separately regulated proteins, galactosyl transferase and α-lactalbumin. Galactosyl transferase is induced by prolactin prior to α-lactalbumin in mouse mammary gland explants (Jagoda and Rillema 1991). Among the various hormones and growth factors secreted into the milk of mammals, EGF has been described to be produced under the control of prolactin (Fenton and Sheffield 1991). Expression of an insulin-like growth factor (IGF) is induced by prolactin and other hormones belonging to the prolactin family (Fielder et al. 1992). By contrast, there is no evidence for such a regulation in bovine mammary tissues (Campbell et al. 1991). The major constituent of the "cropmilk" induced in response to prolactin in pigeons and doves is annexin Icp35 (Hitti and Horseman 1991; Xu and Horseman 1992). The mammalian

Table 2. Prolactin-regulated genes

Tissue	Gene product	Tissue	Gene product
Mammary gland	*Milk proteins*	Liver	PEPCK
	α-Caseins		Synlactin
	β-Casein	Prostate	IGF-I
	WAP		IGF-I receptor
	β-Lactoglobulin		Androgen receptor
	LLP		Mitochondrial aspartate aminotransferase (mAAT)
	Lipid metabolism	Nb2 lymphoma	IRF-1
	Acetyl CoA carboxylase		Myc gene product
	Fatty acid synthase		ODC
	Malic enzyme		Hsp-70-like protein
	Lipoprotein lipase	Uterus	α_2-Macroglobulin
	Lactose synthesis		Uteroglobin
	Lactose synthetase	Ovary	α_2-Macroglobulin
	Galactosyl transferase		Luteinizing hormone receptor
	α-Lactalbumin		
	Glucose carrier		
	Growth factors and receptors		
	IGF-I-binding protein		
	EGF		
	Other proteins		
	Annexin Icp35		
	120-kDa protein		
	Muc-1		
	PIP		

WAP, whey acidic protein; *LLP*, late lactation protein; *IGF-I*, insulin-like growth factor; *EGF*, epidermal growth factor; *PIP*, prolactin-inducible protein; *PEPCK*, phosphoenolpyruvate carboxykinase; *mAAT*, mitochondrial aspartate aminotransferase; *IRF-1*, interferon regulatory factor-1; *ODC*, ornithine decarboxylase.

counterpart of this calcium-dependent, phospholipid-binding protein is also highly expressed in the mammary gland, but is not regulated by prolactin (Horlick et al. 1991). The late-lactation protein (LLP) has been identified as a novel, presumably prolactin-regulated milk protein predominant in the "late milk" of marsupials (Collet et al. 1989, 1991).

In cell lines derived from the mammary gland, three gene products of unknown function have been found to be induced by prolactin. The mammary epithelial cell line HC11 has been shown to accumulate a transcript encoding a 120-kDa protein when treated with prolactin in the presence of the protein synthesis inhibitor cycloheximide (Ball et al. 1988b). This protein has not been characterized further. Muc-1, a highly glycosylated mucin expressed on the surface of mammary epithelial cells has been shown to be induced by prolactin in CID-9 mammary epithelial cells (Parry et al. 1992). Prolactin-inducible protein (PIP) was described as a secreted, prolactin-

inducible protein expressed by human breast cancer cells (Shiu and Iwasiow 1985). The cDNA cloning revealed the identity of PIP with the gross cystic disease fluid protein-15 (GCDFP-15) found in human breast cystic fluid (Murphy et al. 1987; Myal et al. 1991).

The mouse mammary tumor virus (MMTV) is expressed at high levels in lactating mammary glands. Enhancer elements in the long terminal repeat (LTR) have been localized which direct the mammary epithelial cell-specific expression (Mink et al. 1992). There is controversy about a positive contribution of prolactin to the mammary-specific expression of viral messenger RNA (mRNA). One report described a stimulation of MMTV-LTR directed expression by prolactin in transiently transfected human breast-carcinoma cells (Haraguchi et al. 1992). However, in transgenic mice (Mok et al. 1992) or stable transfected mouse mammary epithelial cells (Härtig et al. 1993) no evidence for such a regulation was found.

There are fewer reports about prolactin-regulated genes in other organs. In the liver, prolactin increases the mRNA levels of the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK; Zabala and Garcia-Ruiz 1989) and induces synlactin, a secreted factor that acts synergistically with prolactin on the growth of the pigeon crop sac (Nicoll et al. 1985).

In rat prostate, prolactin enhances the effects of androgens by increasing androgen receptor levels (Prins 1987). In addition, a prolactin-dependent increase of IGF-I message in prostatic stroma together with an increase of IGF-I receptor in the rat prostate was observed, suggesting that prolactin is establishing a paracrine-acting IGF-I system. Citrate production and accumulation have been described to be unique properties of the rat and human prostate. A key regulatory enzyme in this process, the mitochondrial aspartate aminotransferase (mAAT) has been described to be stimulated by prolactin in pig prostate epithelial cells (Franklin et al. 1992).

In Nb2 lymphoma cells prolactin regulates a number of growth related genes (Table 2; de Toledo et al. 1987; Yu-Lee 1990). The interferon regulatory factor IRF-1, a transcription factor which has been found to be inducible by a variety of cytokines (Yu-Lee et al. 1990; Schwarz et al. 1992), belongs to this set of genes and is induced during both early G1 and the G1→S transition (Stevens and Yu-Lee 1992). In the murine T lymphocyte cell line L2 induction of IRF-1 by prolactin at the G1→S transition was also observed. However, the induction of IRF-1 at early G1 was triggered by IL-2, which is necessary for stimulating proliferation in this cell line, and not by prolactin. It is possible that the aberrant expression of PRL-Rs in Nb2 cells (Ali et al. 1991; O'Neal et al. 1991) is responsible for the altered response of this tumor cell line.

In the mesometrial decidua of the uterus (Gu et al. 1992) and in ovary (Gaddykurten and Richards 1991) α2-macroglobulin has been found to be positively regulated by prolactin. This protease inhibitor was previously

described as an acute phase protein induced in liver cells by IL-6. Its regulated expression in the decidua might be important for protecting the mesometrium against extensive tissue damage as a result of trophoblast invasion (Gu et al. 1992). The uteroglobin gene has also been found to be induced by prolactin in the uterus (Randall et al. 1991; Kleis-San Francisco et al. 1993). By inducing the levels of the luteinizing hormone receptor in the corpus luteum, prolactin increases the susceptibility of the organ to respond to that hormone (Gåfvels et al. 1992).

There appears to be no unifying mechanism underlying the induction of gene expression by the hormone. Both direct and indirect effects on transcription initiation and posttranscriptional effects have been described. In the regulation of the β-casein gene expression, both mechanisms might be utilized simultaneously (Eisenstein and Rosen 1988; Doppler et al. 1990; Goodman and Rosen 1990). In this review I will focus only on the mechanism by which prolactin stimulates the initiation of transcription, since there is presently no precise information as to how prolactin influences gene expression at other levels of regulation.

5 Search for Prolactin Response Elements

With the availability of genomic clones of milk protein genes, the identification of regulatory *cis*-acting sequences by gene transfer experiments employing truncated and mutated genes and gene fragments appeared to be straightforward. However, due to an initial lack of transfectable cell lines that maintained the capability to produce milk protein genes, the first successful demonstration of the existence of regulatory elements within these genes came from experiments with transgenic mice (reviewed in Henninghausen 1990; Harris et al. 1990). Mammary gland explant cultures of transgenes harboring whey acid protein (WAP) gene promoter constructs revealed that a prolactin response region was contained within a 2.4-kb promoter fragment of the mouse WAP gene (Pittius et al. 1988; Schoenenberger et al. 1990). The first successful demonstration of prolactin response regions by gene transfer experiments employing mammary epithelial cells was done with a 2.3-kb fragment of the rat β-casein gene promoter (Doppler et al. 1989). The novel developed cell line HC11 (Danielson et al. 1984; Ball et al. 1988b) and the selection of stably transfected cells were found to be crucial for an analysis of a prolactin-dependent transcriptional response. With this system, a fine mapping of the prolactin response region to an element extending from –176 to –82 in the rat β-casein gene promoter was possible (Doppler et al. 1989; 1990 and unpublished data). Similar experiments done with the mouse WAP gene promoter defined a minimal hormone

response region extending form –165 to +24 (Doppler et al. 1991; Jennewein 1992). Using a similar approach, a prolactin-inducible enhancer region was found between –1676 and –1517 in the bovine β-casein gene (Schmidhauser et al. 1990, 1992). In HC11 cells, attempts to simplify the screening procedure for response elements by employing transient transfection assays failed. Both the β-casein and the WAP gene promoters were expressed at least 10 000 fold weaker in transiently transfected cells, irrespectively of which transfection technique was used (Doppler et al. 1991). However, with primary mouse mammary epithelial cells, it was possible to develop a transient transfection protocol (Yoshimura and Oka 1990a), which served to define prolactin response elements in the mouse β-casein gene between –258 and +7 (Kanai et al. 1993), the rabbit WAP gene between –3000 and +490 (Devinoy et al. 1991) and the rabbit αS1-casein gene between –3768 and –3155 (Pierre et al. 1992). In addition to the systems utilizing mammary epithelial cells, a very elegant technique for the screening of prolactin response elements was published (Lesueur et al. 1990, 1991; Ali et al. 1992). Transient cotransfection of CHO cells with PRL-R expression vectors together with reporter constructs containing potential prolactin response regions were performed. The importance of this type of experiment to define PRL-R regions required for intracellular signaling has already been discussed (Sect. 2.2.3). So far, there have been no reports about the utilization of this system in defining prolactin response regions. A potential caveat in that approach is the employment of a nonmammary epithelial cell for studying the regulation of mammary cell-specific regulated genes. In fact, the first nuclear factor with a documented role in the prolactin response is a mammary cell-specific factor (Sect. 6). Gene transfer of a lactogenic hormone response region into living mice by jet injection of DNA was described recently (Furth et al. 1992). Expression of the injected WAP gene promoter construct was detectable in explant cultures of the mammary glands derived from injected mice. Additional work has to be performed to evaluate the advantages of this novel method in comparison to the established techniques.

6 Mammary Gland Factor MGF: A Prolactin-Inducible Nuclear Factor

The relatively precise localization of the prolactin response region of the rat β–casein gene promoter allowed a focused analysis of the nuclear factors binding in vitro to that region (Schmitt-Ney et al. 1991). Only one of the factors found was specific for the mammary gland. Because of this property, the factor was termed MGF (mammary gland factor). Mutation of the MGF

binding site abolished the function of the β-casein gene promoter in responding to the synergistic action of prolactin and glucocorticoids, implicating the importance of this factor in regulating the inducible gene expression of milk protein genes. Binding sites for this factor were found in both α- and β-casein gene promoters (Table 3). A rat mammary gland preparation with highly purified MGF was size-fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). MGF binding activity could be assigned to a region of the gel where a single 89-kDa protein was detectable (Wakao et al. 1992). Binding of MGF in vitro was shown to be dependent on phosphorylation. Incubation with phosphatase abolished binding, whereas treatment with casein kinase II was partially able to restore the binding activity (Schmitt-Ney et al. 1992a).

6.1 Mechanism of Activation

Examination of the MGF binding activities at various stages of the mammary gland development was performed (Schmitt-Ney et al. 1992a,b; K. Garimorth, unpublished). Activity was high during lactation and dropped rapidly in the involuting gland. Restimulation of milk synthesis in the glands of weaning mothers by adding back the suckling pups reactivated MGF activity within 90 min. Unexpected, MGF activity was highest at the beginning of lactation and then dropped markedly within the next days (K. Garimorth, unpublished). By contrast, β-casein production is relatively low at the beginning of lactation and continues to increase over a period of several days. When nuclear extracts of HC11 cells stimulated with prolactin and dexamethasone for 4 days were investigated, only very low levels of a factor with slightly slower mobility than MGF but with indistinguishable binding specificity could be detected (Schmitt-Ney et al. 1992a). Later work (T. Welte, unpublished) revealed a rapid down-regulation of MGF binding activity in these cells by prolonged incubation with prolactin. The factor was maximally induced within 15 min after addition of prolactin. Neither glucocorticoid hormone nor insulin was required for the activation process. Cycloheximide did not block the activation, thus de novo protein synthesis was not required. Genistein and staurosporin, two inhibitors of tyrosine kinase activity, inhibited the activation of the factor by prolactin, indicating the involvement of tyrosine kinases. The factor appeared to be a direct substrate of a tyrosine kinase, since phosphotyrosine-specific antibodies and the phosphotyrosine analog phenylphosphate blocked the binding of MGF in vitro (T. Welte, unpublished). The sequences of the published binding sites for MGF are shown in Table 3.

Table 3. Binding sites for MGF, growth hormone-inducible factor, and STAT family members

Binding site	Inducer	Sequence	Reference
Rat β-casein, proximal site	Prolactin	TTCTTGGAATT	Schmitt-Ney et al. 1991
Rat β-casein, distal site		TTCTTGGGAAA	
Bovine β-casein		TTCTAGGAATT	Wakao et al. 1992
Bovine αS1-casein		TTCTTAGAATT	
Consensus of MGF core site:		TTCTYRGRAYY	
Spi 2.1 gene	Growth hormone	TTCTGAGAAAT	Yoon et al. 1990
Rat α2 macroglobulin (APRE)	IL-6	TTCTGGGAATT	Wegenka et al. 1993
FcγRI (GRR)	INF-γ	TTCTGGGAAAT	Pearse et al. 1993
Ly-6E (GAS)	INF-γ	TTACAGGAATA	Khan et al. 1993
Human *c-fos* (SIE)	EGF, PDGF	TTACGGGAAAT	Hayes et al. 1987

MGF, mammary gland factor; *IL*, interleukin; *INF*, interferon; *EGF*, epidermal growth factor; *PDGF*, platelet-derived growth factor.

6.2 Relationship to Other Inducible Nuclear Factors

The sequence motif of MGF matches the motifs published for a number of growth factor and cytokine inducible proteins. A growth hormone-inducible DNA-binding protein has been identified in rat liver (Yoon et al. 1990). It binds to a growth hormone response region in the serine protease inhibitor (Spi) 2.1 gene. The sequence of the binding region contains a motif highly related to the MGF consensus (Table 3). Induction of the factor was perfomed by injection of human growth hormone into hypophysectomized and hypothyroid male rats. As already mentioned in Sect. 2, human growth hormone has both somatogenic and lactogenic activity. Thus further experiments are required to rule out the possiblity that an activation of PRL-Rs by human growth hormone is responsible for the induction. Very similar binding sites have also been described for the EGF and *sis*-inducible factor (SIF) (Hayes et al. 1987), the interferon-γ inducible transcription factor complexes (Pearse et al. 1993; Wilson and Finbloom 1992; Khan et al. 1993), and the IL-6-inducible acute phase response factor APRF (Wegenka et al. 1993).

Common principles in the mechanism of the activation of SIF, APRF, the factors induced by interferon-α, -β and -γ and other cytokines have been described recently (Schindler et al. 1992; Larner et al. 1993; Ruff-Jamison et al. 1993; Silvennoinen et al. 1993; Sadowski et al. 1993). The well-studied mechanism by which the signal transducer and activator of transcription Stat91 induces transcription serves as a paradigm. This 91-kDa protein belonging to the ISGF3 γ family contains a SH2 domain. The protein is rapidly phosphorylated on tyrosine 701 in response to interferon-γ. This

phosphorylation is necessary for the activation of the factor (Shuai et al. 1993). Only the phosphorylated protein is bound to the γ-interferon response region (GRR) as part of the transcription factor complex. Whether Stat91 alone is sufficient for binding or whether additional peptides are required is presently unknown. Stat91 is also part of some, but not all, of the other transcription factor complexes induced by cytokines and peptide growth factors investigated so far. The site of phosphorylation is presumable a membrane-bound complex of the factor and the activated receptor (David et al. 1993).

MGF appears to belong to the same family of factors. It binds to the same or a closely related DNA recognition sequence and has a similar mode of activation, namely, phosphorylation on phosphotyrosine. Electromobility shift experiments indicate that the mobility of the MGF complex is different from the interferon-γ inducible transcription factor complex but similar to the IL-6 induced factor APRF (T. Welte , unpublished). The molecular cloning of the component(s) of the factors should give further insights into the relationship of MGF to cytokine-induced factors in the near future.

7 Synergy and Interference of Other Signaling Pathways with Prolactin Action

A frequent finding characteristic for prolactin-induced gene expression is the dependence on other activated signaling pathways (Table 4). This appears to be one important control mechanism to restrict a specific prolactin response to appropriate tissues and stages of development.

7.1 Steroid Hormones

Synergistic and antagonistic interactions of prolactin signaling pathways with steroid hormone-dependent cellular responses are frequently observed, especially in the mammary gland (reviewed by Topper and Freeman 1980). Glucocorticoid hormones have in most cases a pronounced synergistic effect on prolactin-dependent milk protein gene expression. The degree of synergism is not the same for all milk protein genes (Ono and Oka 1980; Ray et al. 1986; Puissant and Houdebine 1991). This might contribute to the noncoordinate regulation of milk proteins in the mammary gland. The synergism between glucocorticoid hormones and prolactin also can be observed in the regulation of stable transfected milk protein gene promoter constructs (Doppler et al. 1989, 1991, Schmidhauser et al. 1992), indicating that the two hormones both act on the level of transcription. Again, the mode of synergism was not the same for different milk protein gene regulatory elements. In the case

Table 4. Factors influencing the effect of prolactin on gene expression

	Factor	Effect
Steroids	Glucocorticoids	+
	Progestins	– or +
	Estrogens	+
	Androgens	+
	Thyroxine	+
Peptide factors	Insulin/IGF-1	+
	Growth hormone	+
	EGF/TGF-α	– or +
	TGF-β	–
	IL-2	+
Cellular organization	Cell–cell interactions	+
	Interactions with basement membrane constituents	+
	Establishment of the cellular polarity	+
	Accessability of hormonal receptors	+

IGF, insulin growth factor; *EGF*, epidermal growth factor; *TGF*, transforming growth factor; *IL*, interleukin.

of the rat β-casein gene promoter only in the presence of both hormones an effective activation of transcription of the linked reporter was possible. By contrast, the WAP gene promoter was induced considerably by glucocorticoids alone (Doppler et al. 1991) and the lactogenic hormone enhancer of the bovine β-casein was only weakly dependent on the steroid hormone. In the WAP gene promoter, the glucocorticoid effect alone was found to be variable and appeared to depend on the integration site of the transfected promoter (Jennewein 1992). Mammary gland explant cultures of transgenic mice which harbored WAP promoter constructs juxtaposed to matrix-attachment regions showed a strict dependence on both glucocorticoid hormone and prolactin for induction of the transgene (McKnight et al. 1992).

What is the mechanism of synergy between glucocorticoid hormones and prolactin? Analysis of the kinetics of hormonal induction of the endogenous β-casein gene and a stable, transfected β-casein gene promoter construct revealed a fast response to prolactin and a slow response to glucocorticoid hormone (Doppler et al. 1991). The delayed effect of glucocorticoid hormones and the absence of classical glucocorticoid binding sites in the hormone response region of the β-casein gene promoter pointed to an indirect effect, requiring de novo synthesis of proteins. Indeed, such requirement has been demonstrated for the mouse β-casein gene expression (Yoshimura and Oka 1990b). Investigations performed with purified rat liver glucocorticoid receptor preparations (Welte et al. 1993) have identified nonclassical glucocorticoid receptor binding sites in the hormone response regions of the WAP gene promoter and the β-casein gene promoter. These sites are related to the delayed

secondary glucocorticoid receptor binding sites found in the regulatory region of the α2u-globulin gene (Chan et al. 1991) and might also be involved in the delayed response of the milk protein genes to glucocorticoids.

Progesterone has been found to act negatively or positively on the regulation of prolactin-dependent genes, depending on the tissue and on the developmental stage. In the mammary gland the hormone is described as an inhibitor of prolactin-induced milk protein synthesis at the end of pregnancy, when progesterone levels are high (Sankaran and Topper 1988; Jahn et al. 1989). A nuclear factor induced by progesterone which binds to the mouse β-casein gene promoter has been identified (Lee and Oka 1992a,b). Mutational analysis revealed a functional role of this factor in repressing the lactogenic hormone response of the promoter. It is possible that in addition to these factors, the activated progesterone receptor acts directly on the β-casein gene promoter. The recently identified glucocorticoid receptor binding sites (Welte et al. 1993) might also be utilized by the progesterone receptor, thereby antagonizing the action of the glucocorticoid receptor. In contrast to the negative effect of progesterone on the terminal differentiation of the gland, in some cases progestins are believed to increase the proliferative effects of prolactin on mouse mammary epithelium cells, human tumor cell lines, and pregnancy-dependent mouse mammary tumors (Vonderhaar and Biswas 1987; Sakai et al. 1990). A servomechanism of prolactin and progesterone has been proposed to regulate uteroglobin expression in the uterus of rabbits (Chilton et al. 1988). The positive effects of prolactin on uteroglobin induction is thereby amplified by a progesterone-dependent increase of the PRL-R levels.

Numerous effects of other steroid hormones on prolactin action have been described; many of them appear to occur at the level of modulation of receptor expression (Ormandy and Sutherland 1993). Androgens act synergistically with prolactin in the human tumor cell line T47-D on the induction of PIP (Murphy et al. 1987) and increase PRL-R binding sites in a number of tumor cell lines (Ormandy et al. 1992). Estrogens are potent inducers of the PRL-R in the liver of female rats (Posner et al. 1974). This early observation greatly facilitated the cloning of the receptor (Boutin et al. 1988). α-Lactalbumin but not α-casein synthesis was enhanced by treatment of hypophysectomized pregnant mice with a combination of corticosterone and thyroxine (Thordarson et al. 1992), indicating that the expression of α-lactalbumin has a selective requirement for thyroid hormone.

7.2 Peptide Factors

The synthesis of milk protein genes is depende... on insulin (Topper and Freeman 1980). IGF-I can be substituted for insulin in inducing milk protein

synthesis (Prosser et al. 1987). Whether the effect is mediated by the insulin receptor or the receptor for IGF-I is unknown. The mode of synergy between prolactin and insulin is presently unclear. Insulin was not required for the transcriptional effects of prolactin on the expression of stably transfected milk protein gene promoter constructs (Doppler et al. 1989) or for the activation of MGF (T. Welte , unpublished).

EGF has been shown to inhibit lactogenic hormone-dependent milk protein gene synthesis and transcription (Taketani and Oka 1983; Hynes et al. 1990; Doppler et al. 1991). Expression of transforming growth factor α (TGF-α), which binds to the same receptor as EGF, was also blocking the transcriptional induction of the milk protein β-casein (Hynes et al. 1990). A blockade of MGF induction by long-term exposure of cells to prolactin and EGF was reported in HC11 cells (Schmitt-Ney et al. 1992b). However, a short-term exposure of the same cells to EGF did not inhibit the transient activation of MGF by prolactin (T. Welte, unpublished), indicating that the inhibitory action of EGF was not a result of a direct interference with a prolactin-induced signaling pathway. Cultivation of HC11 cells in EGF had a positive effect on lactogenic hormone action, but only when the growth factor was present in the medium prior to the addition of lactogenic hormones and was removed concomitantly with the addition of homones (Taverna et al. 1991).

TGF-β blocked the lactogenic hormone induced synthesis of β-casein (Mieth et al. 1990; Robinson et al. 1993). The effect was posttranscriptional and did not affect the level of casein mRNA in explant cultures. Since expression of TGF-β2 and TGF-3 was found to be high during pregnancy but reduced at the onset of lactation, it was hypothesized that these factors are physiological inhibitors of an accumulation of milk proteins prior to term (Robinson et al. 1993).

In the T-cell line L2, IL-2 has been found to be a comitogen, together with prolactin (Clevenger et al. 1990). Analysis of the expression pattern of cell cycle-related proteins after stimulation with IL-2, prolactin, or both hormones led to the preposition of a sequential model of T-cell activation, requiring IL-2 for the early G1→G1 transition and prolactin, as a progression factor, for a late G1→S phase transition (Clevenger et al. 1992).

7.3 Cellular Organization

Many of the cell types which have an intrinsic capability to respond express prolactin responsive genes only under special culture conditions. For mammary epithelial cells, the culture conditions which are required for an efficient synthesis and secretion of milk protein genes have been extensively characterized (Strange et al. 1991 and references therein). Cell–cell interac-

tions promoted by cocultivation of nonepithelial with epithelial cell lines, cell interactions with basement membrane constituents and/or establishment of the cellular polarity and the accessibility of hormone receptors have been found to promote hormone-dependent milk protein synthesis. Effects on transcription (Schmidhauser et al. 1992), posttranscriptionally regulated message accumulation (Eisenstein and Rosen 1988; Zeigler and Wicha 1992), and secretion (Strange et al. 1991) of milk protein genes were observed. The epithelial cell lines 31E (Reichmann et al. 1989, Strange et al. 1991) and HC11 (Ball et al. 1988b) did not dependent on exogenously added basement membrane components, indicating that they are able to synthesize these components by themselves. Cultivation of the 31E cells in a filter chamber allowed the formation of electrically tight polarized epithelial cell layers. They were only responsive to prolactin added to the basal chamber, indicating a restriction of PRL-R expression to the basal cell surface. The cell exhibited a polarized secretion of laminin into the lower chamber and of β-casein into the apical chamber (Strange et al. 1991).

8 Dysregulation of Prolactin-Dependent Gene Expression

Until recently, very little was known about the physiological mechanisms by which prolactin activates gene epression. Thus, only a limited evaluation of aberrant regulatory mechanisms was possible so far.

8.1 Effects of Oncogene Expression

The availability of prolactin-responsive clonal cell lines allowed the assessment of effects induced by the expression of transfected or infected oncogenes. The only reported positive effect on the response to lactogenic hormones was observed by introducing *v-myc* into a mouse mammary epithelial cell line (Ball et al. 1988a). Nuclear extracts of tumor cells derived from WAP-*myc* transgenes exhibited high constitutive levels of a factor which migrated at a different position in comparison to MGF found in pregnant mice (Happ et al. 1993). Whether this factor exhibits the same binding specificties as MGF was not shown. Experiments with *v-myc* infected HC11 cells did not show an effect on lactogenic hormone dependent β-casein gene promoter activity (Jehn et al. 1992). Thus, further experiments are required to establish the role of *myc* oncogene products in the cellular response to prolactin.

Transfection or infection with an activated *Ha-ras* oncogene consistently decreased the response to lactogenic hormones (Hynes et al. 1990; Jehn et al. 1992; Happ et al. 1993). Evidence for two different sites of the inhibitory

action has been presented. In *Ha-ras* transfected HC11 cells, no MGF was detectable after a 2-day induction with lactogenic hormones (Happ et al. 1993), implicating an effect of activated p21 ras on the prolactin signaling. Cellular synthesis of p79 gag-raf, an oncogene product implicated to act downstream of p21ras, had similar effects. However, before definite conclusions can be drawn, experiments have to be done which compare the effects of *Ha-ras* and *v-raf* on the strong transient activation of MGF by prolactin. Another report describes a negative effect of *Ha-ras* on the glucocorticoid receptor-mediated signaling by increasing the levels of the transcription factor AP-1 (Jehn et al. 1992). A similar mechanism was proposed by the same authors to be responsible for the impaired lactogenic hormone response of *v-src* and *mos* expressing HC11 cells. It is possible that the inhibitory effects of EGF are also mediated by p21 ras, since stimulation of the EGF receptor results in an increase of the active p21 ras-GTP (Grunicke and Maly 1993). An additional potential connection between p21 ras and EGF action are the observed elevated TGF-α levels in the culture medium of *Ha-ras* transfected HC11 cells (Hynes et al. 1990), which suggests the establishment of an autocrine loop by the activated oncogene.

Expression of the oncogenes *int-2*, activated rat *neu*, or T-antigen in HC11 cells did not affect the response to prolactin (Hynes et al. 1990; Wolff et al. 1992; Happ et al. 1993). However, all transfectants acquired the phenotype of transformed cells, indicating that transformation of cells per se was not sufficient to change the response to lactogenic hormones.

8.2 Aberrant Prolactin Signaling in Tumors

The role of dysregulated prolactin-dependent pathways in tumor formation was addressed mainly by studying mammary gland tumors. Measurement of prolactin hormone levels in the serum of tumor patients (Di Carlo et al. 1988; Maddox et al. 1992; Adams 1992), and the determination of PRL-R binding sites in tumors and tumor cell lines (Shiu 1979; Bonneterre et al. 1987; DiCarlo et al. 1988; Holtkamp et al. 1988; Bonneterre and Peyrat 1989; De Placido et al. 1990) were performed. The studies revealed no conclusive evidence for aberrant prolactin signaling in human tumors. In addition, pharmacological blockade of prolactin secretion (Kleinberg 1987) was not effective in repressing the growth of human tumors. The novel insights in the regulation of prolactin gene expression should stimulate a reevaluation. Instead of the insensitive screening for binding sites in tumors with radioactively labeled hormone, the availability of the cloned receptor will allow a measurement of receptor mRNA abundancy and structure and will facilitate the development of receptor-specific antibodies. It is possible

that these studies will reveal the existence of aberrant human receptor forms. In the rat tumors, two cases with aberrant receptor expression already have been documented. First, Nb2 lymphoma cells have a truncated version of the long form of the receptor (Ali et al. 1991). Second, an increased expression of the PRL-R without recognizable change in the structure of the protein was detected in a Moloney murine leukemia virus induced rat thymoma. The activation of the receptor gene was the result of the insertion of the 5' long terminal repeat in front of the PRL-R (Barker et al. 1992). Thus in T-cells, the PRL-R may function as an oncogene. The development of prolactin receptor-specific antagonists (Davis and Linzer 1989b; Fuh et al. 1993) should facilitate a selective blockade of the PRL-R. The pharmacological blockade of prolactin secretion done so far is only partially effective in this respect, since it does not inhibit the synthesis of human growth hormone (Köbberling 1983), which can also bind and activate the PRL-R.

An investigation of alterations of prolactin-dependent gene expression in human tumors has not been conducted yet. The analysis of the protein composition of cyst fluids revealed a change of the expression pattern of milk proteins in samples of breast tumor patients (Vizoso et al. 1992). Whether this change was due to an altered response to prolactin is unknown. GCDEFP-15 and Muc-1, two prolactin-inducible proteins (Sect. 4), are frequently found in tumors and have already been used as tumor markers before their regulation by prolactin was discovered. Again, the potential role of prolactin in causing the high levels of these proteins in some tumors is unclear. In the future, a better understanding of the intracellular signaling employed by prolactin and the cloning of the gene or the genes encoding the prolactin responsive nuclear factor MGF will facilitate the detection and understanding of a dysregulated expression of prolactin-dependent genes in tumor cells.

9 Summary and Conclusions

The molecular characterization of the receptor for prolactin and the identification of a nuclear factor involved in the transcriptional activation of prolactin dependent genes have greatly increased our understanding about the mechanism by which this multifunctional hormone prolactin activates gene expression. At least in the activation of milk protein gene expression, the following sequence of events appears to be initiated by the binding of prolactin to its receptor: (1) ligand-induced receptor dimerization; (2) activation of one or more cytoplasmic tyrosine kinases attached to the receptor; (3) phosphorylation of the prolactin-inducible nuclear factor MGF on tyrosine; (4) binding of MGF to its response element on the DNA. This mode of action

is very similar to the mechanisms employed by cytokine and growth factor receptors. In addition to the similarities in the mode of action, the receptors and nuclear factors are also structurally related. There is significant sequence homology between the PRL-R, the GH-R, the majority of cytokine receptors, and the interferon receptors. Also the DNA recognition motifs utilized by MGF and nuclear factors induced by interferons, cytokines, and peptide growth factors are highly conserved. This points to an evolutionary relationship of the signaling pathways employed by peptide growth factors, prolactin, growth hormone, a number of cytokines, and the interferons.

The multifunctional activity of prolactin has its biochemical correlate in the developmental and stage-specific control of the receptor and receptor subtypes and in the differential dependence on the activation of other, mostly steroid hormone-controlled signaling systems. It is also likely that there are cell type-specific differences in the signaling pathways coupled to the PRL-R. An additional way to control different physiological actions by the same receptor signaling system is achieved by the temporal and spatial regulation of expression of multiple genes encoding proteins which bind with high affinity to the PRL-R. With the PRL-R and the prolactin-inducible nuclear factor MGF two proteins involved at the beginning and at the endpoint of prolactin-dependent gene regulation have been identified. The further molecular characterization of these proteins will facilitate a biochemical approach to identify other cellular regulators important for the action of prolactin.

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