ADVANCES IN MOLECULAR AND CELL BIOLOGY

Series Editor: E. EDWARD BITTAR
Guest Editor: MICHAEL WHITAKER

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CELL CYCLE

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CELL CYCLE

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PREFACE

There has been an enormous advance in our understanding of the regulation of the cell division cycle in the last five years. The leap in understanding has centered on the cell cycle control protein $p34^{cdc2}$ and its congeners and on the cyclins. The most important insight to emerge has been that cell cycle control mechanisms and their participating proteins are very well-conserved through evolution. This has created a spectacular growth in knowledge as data from one organism have been readily applied to another. In this volume, there are sea urchin and frog eggs, as well as mammalian cells and yeast. There is also an illustration of how fruitful the genetic approach can be in other organisms than yeast with a chapter on *Aspergillus nidulans*.

The cell cycle kinase has been well-characterized and has also been well-exposed in numerous proceedings volumes and collections. In this issue of Advances in Molecular Cell Biology, the cell cycle kinase is ever present, but in the early chapters it has a supporting role. Center stage are the regulatory mechanisms that control the kinase. The contribution that the centrosome (the organelle of cell division) makes to cell cycle regulation are described. The part played by calcium and calcium-controlled regulatory proteins is emphasized. The importance of phosphatase as well as kinase activity to cell cycle regulation is stressed. The last words are reserved for the mitotic kinase: the last chapters describe its effects and its regulation in cell-free systems.

Michael Whitaker
Guest Editor

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CENTROSOMES AND THE CELL CYCLE

Greenfield Sluder

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ABSTRACT

Centrosomes, the ensembles of structures that define the poles of the mitotic apparatus, play pivotal roles in almost all mitotic events. Centro-

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somes nucleate microtubules and they precisely double, or reproduce, before mitosis. If mitosis is to be normal, centrosomal events must be tightly coordinated with nuclear events throughout the cell cycle. This chapter reviews a poorly understood facet of centrosome biology: the interrelationship between centrosomes and the cell cycle. This relationship is multifaceted. The cycle of the maturation promoting factor activity that drives the nuclear cell cycle may also drive the cyclical change in microtubule-nucleating activity of centrosomes. However, the events of centrosome reproduction are not controlled by, and are thus independent of, nuclear activities and the cycle of the maturation promoting factor activity. In addition, centrosomes, through the microtubules they nucleate, play an important role in the mechanisms that determine when the dividing cell will execute the metaphase-anaphase transition point of the cell cycle.

I. INTRODUCTION

Mitosis depends critically upon centrosomes, the ensembles of structures that form the poles of the mitotic spindle. Although the ultrastructure of centrosomes can vary greatly from one organism to the next, they all have two well-established activities: they nucleate essentially all the microtubules of the spindle, and they precisely double, or reproduce, before the cell enters mitosis. Since centrosomes define the essential bipolarity of the division process (Mazia, 1987), the cell must precisely coordinate the doubling of the interphase centrosome with nuclear events during the cell cycle and limit the number of centrosomes it contains at the onset of mitosis to two and *only* two. The penalties for mistakes at the centrosome level are abnormal spindle assembly and inappropriate chromosome distribution during mitosis.

This chapter provides a brief introduction to centrosomes and discusses an important but still not well-understood facet of centrosome biology: the interrelationship between the centrosome cycle and the nuclear cell cycle. The studies reviewed here indicate that this interrelationship appears to be multifaceted. Not only may the microtubule nucleating capacity of centrosomes be driven by the kinase activities that control the nuclear cell cycle, but centrosomes may also play a role in the progression of the cell through interphase and mitosis. Although there is much to be learned from the study of centrosomes in lower organisms, especially those suitable for genetic analysis, our discussion will be restricted to centrosomes of higher animal cells.

A. The Centrosome

Centrosome Ultrastructure

At any given point in the cell cycle, the ultrastructure of higher animal centrosomes, as seen by thin section analysis of fixed whole cells, varies slightly from organism to organism. Also, isolation or cell lysis may influence the apparent ultrastructure of centrosomes (see Bornens et al., 1987; Bornens, 1992 for examples of the ultrastructure of isolated centrosomes). Typically the centrosome consists of a pair of orthogonally arranged centrioles associated with amorphous, osmiophilic pericentriolar material into which the microtubules of the aster and central spindle are inserted. In cross-section the centrioles have a characteristic pinwheel arrangement of nine triplet microtubules embedded in an electron dense matrix that is spatially distinct from the pericentriolar material (Fais et al., 1986). This cylinder of triplet microtubules has within its lumen a number of structures, such as linkers between the triplets, a cartwheel structure at the proximal end of the centriole, twisted fibers, and sometimes a vesicle. In some cell types conical projections called basal feet emanate from the outer wall of the older of the two centrioles, and microtubules insert into the globular tips of these structures (for reviews of centriole and centrosome ultrastructure see Fulton, 1971; Stubblefield and Brinkley, 1967; Rieder and Borisy, 1982; Wheatley, 1982; Vorobjev and Nadezhdina, 1987). In cells that have a primary cilium or a motile cilium, the older of the two centrioles organizes the axoneme (Rieder and Borisy, 1982; Wheatley, 1982).

In some cells the pericentriolar material is distributed primarily around the older of the two centrioles. The younger centriole acquires a full complement of pericentriolar material when it separates from its parent and starts to form a daughter centriole (Rieder and Borisy, 1982; Vorobjev and Chentsov, 1982). In other types of cells the pericentriolar material appears to be localized around the proximal end of both centrioles (Bornens et al., 1987). In some early cleavage stage embryos, the pericentriolar material surrounds both centrioles (Sluder and Rieder, 1985a).

In a number of cultured cell types, denser aggregates of osmiophilic material, called pericentriolar satellites, are seen within or in close proximity to the pericentriolar material. Although some reports suggest that these satellites are a condensed form of the pericentriolar material, relatively recent work indicates that they may be concentrations of the

protein centrin and do not nucleate microtubules (Baron and Salisbury, 1987). Even though the pericentriolar material often appears amorphous in thin section and in negative stain preparations, its fibrogranular appearance suggests that it may have a definite substructure of molecular dimensions. Mazia (1987) has proposed that the centrosome is composed of one or more folded fibers with microtubule-nucleating sites along their lengths.

The pericentriolar material acts as the centrosomal microtubule organizing center (MTOC) during interphase and mitosis. When permeabilized cells are exposed to exogenous tubulin, microtubules are assembled from the pericentriolar material, not the centrioles (Snyder and McIntosh, 1975; Gould and Borisy, 1977). When separated from the centrioles, the pericentriolar material *in vivo* appears to organize a spindle pole during mitosis (Brenner et al., 1977; Berns and Richardson, 1977; Keryer and Borisy, 1984; Sellitto and Kuriyama, 1988). Although the full molecular composition and structure of the microtubule-nucleating sites in the pericentriolar material are not fully understood, recent genetic and localization studies show that the protein gamma tubulin, present in trace amounts, plays a necessary role in microtubule nucleation (Oakley et al., 1990; Stearns et al., 1991). An intriguing question is how the gamma tubulin subunits are arranged to organize the 13 protofilaments of each microtubule.

Centrosome Activities: Microtubule Nucleation

Centrosomes nucleate radial arrays of microtubules throughout the cell cycle. During interphase these microtubules are long and extend throughout the cell. As the cell is about to enter mitosis these microtubules become shorter, sometimes more numerous, and the switch between growth and shortening of their tips occurs more frequently in response to changes in the cytoplasmic environment (Reviewed in Salmon, 1989; Vandre and Borisy, 1989). In telophase the number of centrosomal microtubules drops, and an interphase array is again established.

The average length of astral microtubules varies between mitosis and interphase, and the number of microtubules nucleated by each centrosome varies with the cell cycle. The number of microtubules that a centrosome can nucleate, as determined by microtubule assembly in lysed cell models exposed to 6S tubulin, is maximal at the onset of mitosis and drops when the cell exits from mitosis (Snyder and McIntosh,

1975; Kuriyama and Borisy, 1981b; Snyder et al., 1982). The control of the microtubule-nucleating capacity of centrosomes during the cell cycle could, in principle, be due to the cyclical accumulation and dispersal of the pericentriolar material or by changing the activity of the existing nucleating sites. A number of studies indicate that the extent of microtubule assembly may be controlled in both ways. The amount of osmiophilic material surrounding the centrioles increases as the cell comes into mitosis, suggesting a recruitment of subunits into the centrosomal MTOC (Snyder and McIntosh, 1975). Other studies show that the *in vitro* microtubule-nucleating capacity of the centrosome correlates with the phosphorylation of centrosomal sites. Also, pretreatment of lysed cell models with an antibody that recognizes phosphoepitopes or phosphatases will block centrosomal microtubule assembly (Vandre et al., 1984; Centonze and Borisy, 1990). However, the phosphorylation sites and their role in the formation of microtubule-nucleating structures remain unknown. If reversible phosphorylations control the microtubule-nucleating capacity of the centrosome, the rise and fall of $p34^{cdc2}$ kinase activity that controls nuclear events during the cell cycle (reviewed in Maller, 1991) may coordinate centrosomal microtubule nucleation with the cell's entry into and exit from mitosis (Ohta et al., 1993).

Centrosome Activities: The Reproduction of Centrosomes

At the end of mitosis each daughter cell inherits a single centrosome, and by the onset of the next mitosis each daughter contains just two centrosomes. This precise doubling of the centrosome in preparation for mitosis is called centrosome reproduction. Stated in broad terms, this process consists of a separation of the two centrioles, the assembly of daughter centrioles at right angles to their parents and the splitting of the centrosome as a whole into two daughters, each with a centriole pair that moves to opposite sides of the nucleus before nuclear envelope breakdown.

As the cell traverses G1, the centriole pair loses its orthogonal arrangement (Kuriyama and Borisy, 1981a). Daughter centrioles are first seen in late G1 or at the onset of DNA synthesis with the appearance of short annular centrioles at right angles to and separated slightly from the proximal end of each mature centriole (Robbins et al., 1968). These daughter centrioles, often called procentrioles, elongate during S and G2 and reach their mature length in mitosis or the following G1 (Kuriyama and Borisy, 1981a). Although the way in which procentriole formation

is patterned and spatially positioned is a complete mystery, ultrastructural studies of basal body assembly in lower forms suggest that the process starts, not surprisingly, with the formation of specific precursor structures (Dippel, 1968; Gould, 1975). If such precursor structures for centrioles exist in higher animal cells, it would be of great interest to determine how far in advance of procentriole formation they are assembled.

In cultured cells the centrosome as a whole generally splits at a variable time in G2, with pairs of mother–daughter centrioles going to each daughter centrosome (Aubin et al., 1980). For sea urchin eggs, the experimental material used in many of the studies reviewed here, the centrioles separate and duplicate as the centrosome flattens during telophase (Paweletz et al., 1984; Schatten et al., 1986). These eggs have no G1 phase of the cell cycle, and DNA synthesis starts in telophase (Hinegardner et al., 1964). In G2 the flattened centrosome splits, and the daughter asters move to opposite sides of the nucleus.

When assigning a point in the cell cycle at which the centrosome reproduces, for any experimental system, we must bear in mind that the first visible manifestations of centriole duplication and centrosome splitting probably mark points near the end of these reproductive processes. The molecular doubling of centrosomal components and the assembly of the precursor structures must have occurred at earlier times in the cell cycle.

II. THE INTERRELATIONSHIP BETWEEN CENTROSOMES AND THE CELL CYCLE

A. Control of Centrosome Reproduction

The cell must tightly control the number of centrosomes it contains at the onset of mitosis and precisely coordinate the events of centrosome reproduction with nuclear events. In searching for the mechanisms that control centrosomal events, we can look to limits that are imposed by the structures or molecules of the centrosome itself and controls imposed by changing cytoplasmic conditions. Control of centrosome reproduction appears to be exercised at both levels.

Functional analysis of centrosome reproduction in sea urchin zygotes indicates that there is a counting mechanism within each centrosome that limits the number of daughters that can arise from the parent centrosome. Since a complete description of this analysis is beyond the scope of this

review, only the outlines and conclusions of this work will be covered here (for a full description of this work see Mazia et al., 1960; Sluder and Begg, 1985; reviewed in Sluder, 1990). The key finding is that it is possible to experimentally manipulate the reproductive capacity of centrosomes. When mitosis is prolonged by any of several independent methods, the two spindle poles split during mitosis to yield four functional poles that have a normal appearance but half the normal reproductive capacity; they do not double before the next mitosis and, therefore, four monopolar spindles are assembled (Figure 1). By functional criteria the centrosome of each monopolar spindle has normal reproductive capacity. If the monopolar mitosis is longer than normal, the centrosome will split to give two functional spindle poles that have half the normal reproductive capacity and yield monopolar spindles at the following mitosis. Thus, a centrosome can subdivide once during a prolonged prometaphase and requires a replicative event to return to its full reproductive capacity.

These observations indicate that the reproduction of a spindle pole cannot simply be the subdivision of the centrosomal MTOC, because such a fission mechanism should always produce two smaller centrosomes, each of which should have the reproductive capacity of the parent pole. The phenomena observed in these studies are best explained by postulating that the normal centrosome is organized around two *seeds* or *determinants*, which we call polar organizers, whose splitting and duplication determine the behavior of the visible aster (reviewed in Sluder, 1990). Splitting of the polar organizers is the event that allows the two halves of the centrosome to physically separate from each other, and duplication is the assembly of a new part or second half that remains associated with its parent through the next mitosis. The observation that splitting can occur during a prolonged prometaphase suggests that this event is programmed to occur at the expected time of telophase and is not linked to the metaphase–anaphase transition point. The duplication event, however, does not occur until the cell has gone through this transition point and has finished mitosis.

At the present time we cannot identify the polar organizers in morphological or molecular terms; we can only characterize their behavior by functional criteria. The generative nature of their reproduction would most easily be understood if they were composed, at least in part, of DNA or RNA. In this regard, a number of studies have arguably shown the presence of both nucleic acids in basal bodies of algae and protozoans (Hartman et al., 1974; Dippell, 1976; Berns et al., 1977; Berns and

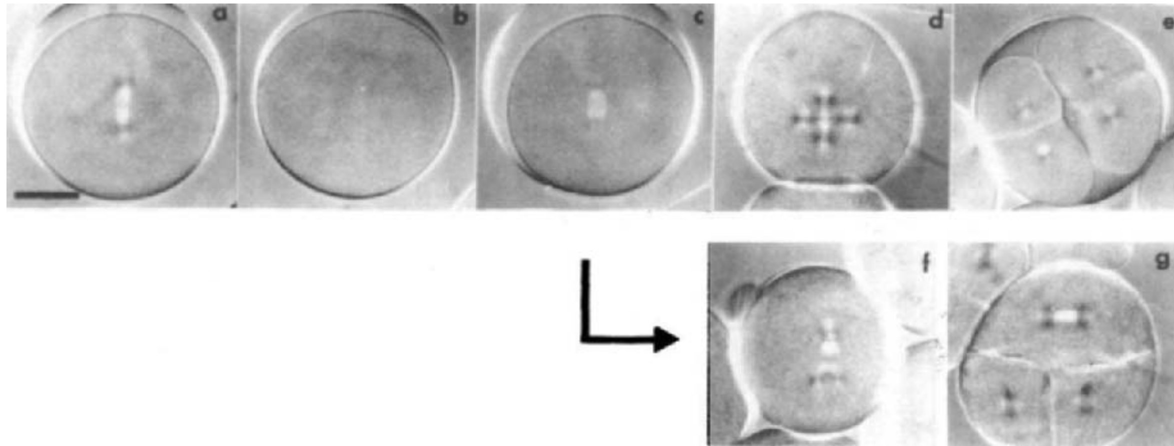


Figure 1. Induction and development of monopolar spindles. a. Prometaphase of first mitosis. b. Immersion in mercaptoethanol to prolong prometaphase. c. Recovery of spindle after removal of mercaptoethanol. d. Later stage of recovery, tetropolar spindle at metaphase. e. Zygote has cleaved directly into four and forms monopolar spindles at second mitosis. f. Alternate mode of recovery; tripolar spindle. g. Zygote has cleaved directly into three; the two daughters that receive split and separated poles form monopolar spindles at second mitosis. The daughter that receives split but unseparated pole forms functional bipolar spindle of normal appearance. 10 μm per scale division.

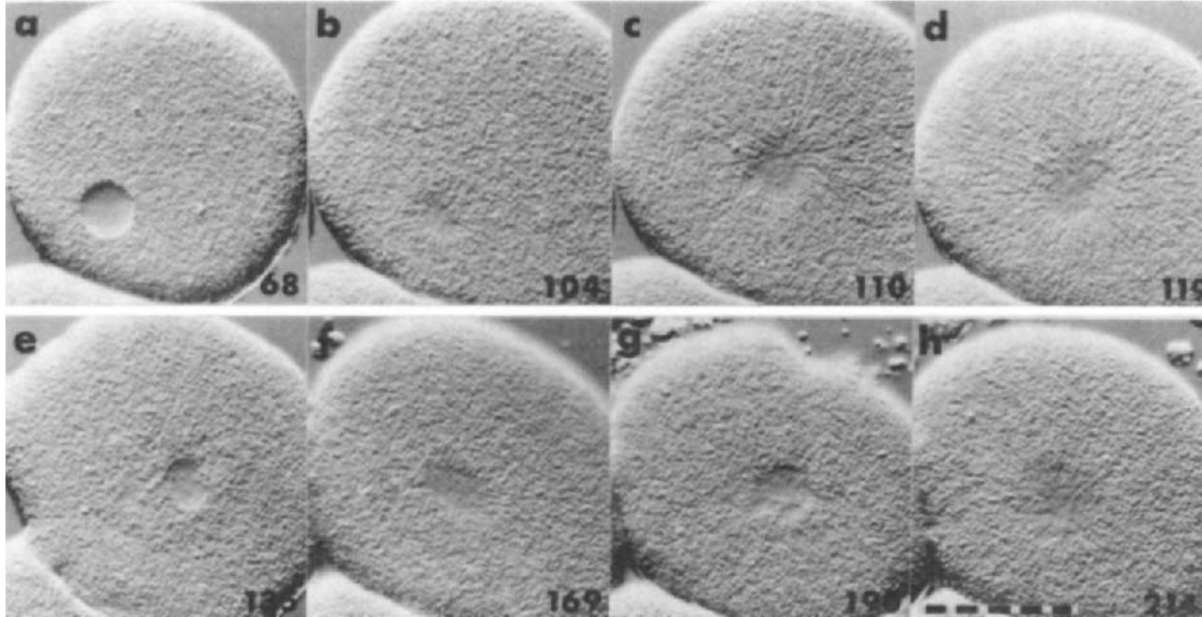


Figure 2. A centrosome without centrioles does not double between mitoses. a. Prophase of first mitosis. b. First mitosis; a single monaster is assembled. c. Later in first mitosis the monaster is more robust. d. Telophase of first mitosis; karyomeres have formed. e. Interphase; the karyomeres have fused into a single nucleus. f. Second mitosis; a single monaster is assembled. g. Interphase; the karyomeres have partially fused to form several micronuclei. h. Third mitosis; a single monaster is assembled. Minutes after fertilization are shown in the lower corner of each frame. 10 μm per scale division.

Richardson, 1977; Heidemann et al., 1977; Peterson and Berns, 1978; Hall et al., 1989; Johnson and Rosenbaum, 1990). However, even if nucleic acids are present in centrioles, these informational molecules seem not to play a functional role in centrosome reproduction (Klotz et al., 1990).

To gain insight into the nature and location of polar organizers, we examined the ultrastructure of centrosomes with full and half-reproductive capacity (Sluder and Rieder, 1985a). We found that centrosomes with half the normal reproductive capacity have only one centriole, while those with full reproductive capacity contain two centrioles. In addition, we have demonstrated for sea urchin and starfish zygotes that a centrosomal MTOC without centrioles does not double between mitoses (Figure 2) (Sluder et al., 1989a; Sluder et al., 1989b). These results are not peculiar to egg systems since cultured cells from which the interphase centrosome has been removed will reform a cytoplasmic MTOC without centrioles. These MTOCs do not double despite the initiation of DNA synthesis and cell growth that should be sufficient to allow entry into mitosis (Maniotis and Schliwa, 1991).

Without belaboring the controversy over the role of centrioles in centrosomes here, our working model is that each polar organizer is spatially and mechanically associated with a centriole in cells that have centrioles. Acentriolar cell types may have polar organizers, but do not have an array of nine triplet microtubules to signal their location (reviewed and discussed in Sluder, 1990).

B. Coordination of Centrosomal Events with Nuclear Events in the Cell Cycle

Role of Nuclear Activities in Centrosome Reproduction

During the normal cell cycle the reproduction of the interphase centrosome is tightly coordinated with nuclear events. For example, the formation of visible procentrioles normally occurs at the onset of DNA synthesis (Robbins et al., 1968; Rattner and Phillips, 1973; Kuriyama and Borisy, 1981a) and the centrosome as a whole does not split until G_2 (Aubin et al., 1980). These observations raise the possibility that nuclear activities could be an obligatory part of the regulatory pathway for centrosome reproduction.

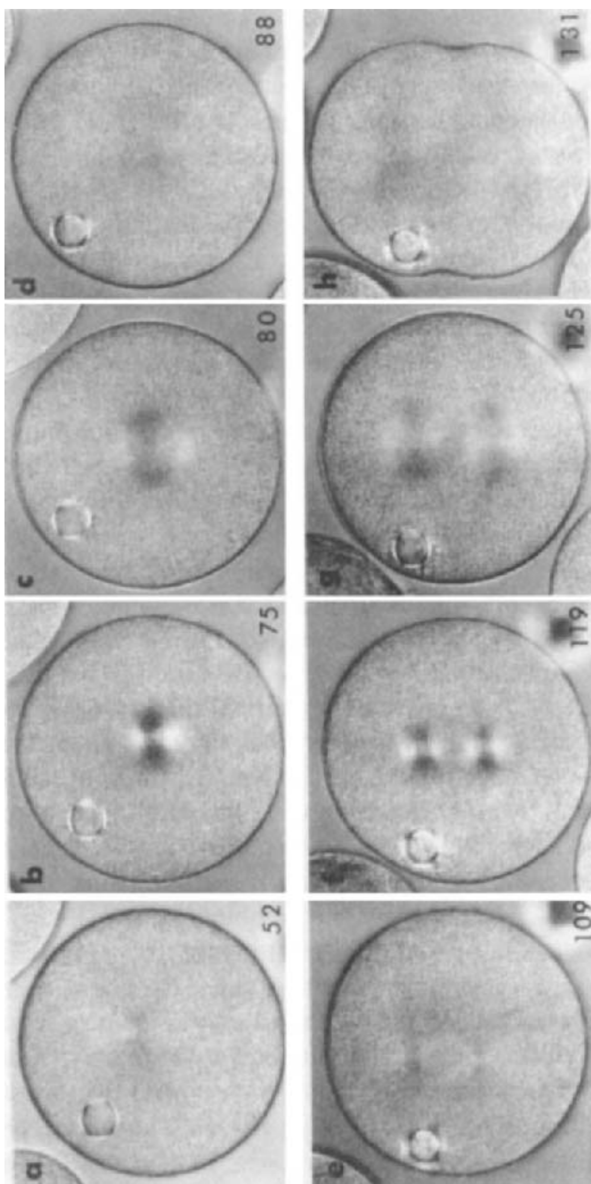
In principle, the involvement of the nucleus in the control and execution of centrosome reproduction could be either direct or indirect. For

example, the cell might require newly synthesized transcripts or their translational products at each cell cycle to assemble centrosomal structures. Also, centrosome reproduction could depend on *signals* from the nucleus (Kuriyama and Borisy, 1981a) that hypothetically originate with the initiation or completion of a nuclear event (Hartwell and Weinert, 1989). Such direct control of centrosome assembly through obligate links to nuclear activities could provide the cell with a logical way to coordinate centrosomal events with nuclear events and control the number of centrosomes formed. Alternatively, new nuclear transcripts or *signals* from the nucleus might not be necessary at each cell cycle. The cell could accumulate nonlimiting pools of centrosomal subunits; the number of new centrosomes formed from these pools would be entirely under cytoplasmic control.

A clear experimental demonstration of the interrelationship between nuclear activities and centrosome reproduction has been difficult to achieve with cultured cells. Since these cells need to grow before they become committed to divide, inhibition of nuclear transcription may block centrosome reproduction by arresting the cell cycle at a point where the cell is not prepared to duplicate these structures. Thus, it is not surprising that studies using cultured cells to examine centrosome reproduction in the absence of nuclear activities have yielded conflicting results and conclusions. For example, the inhibition of DNA synthesis by a variety of agents does not prevent the formation or elongation of daughter centrioles in cultured cells (Rattner and Phillips, 1973; Kuriyama and Borisy, 1981a). However, cells physically enucleated with cytochalasin or chemically enucleated with actinomycin D do not form procentrioles or reproduce their centrosomes (Kuriyama and Borisy, 1981a; Reich et al., 1962; DeFoor and Stubblefield, 1974).

To circumvent the problem of growth limits on progress through the cell cycle, we used sea urchin zygotes to examine the role of the nucleus in centrosome reproduction. Indeed, some early reports suggested that the nucleus may not be required for duplication of asters in sea urchin zygotes (Ziegler, 1898; Lorch, 1952).

We used a micropipette to physically remove the nucleus in prophase of first mitosis leaving only one centrosome in the cell (Sluder et al., 1986). We then followed each enucleated egg with the polarizing microscope to examine changes in astral morphology and determine if the aster reproduced. In all cases, we found that the single remaining aster repeatedly doubled in a precise 1-2-4-8 fashion (Figure 3). In those preparations allowed to progress further, the asters continued to double. Also,



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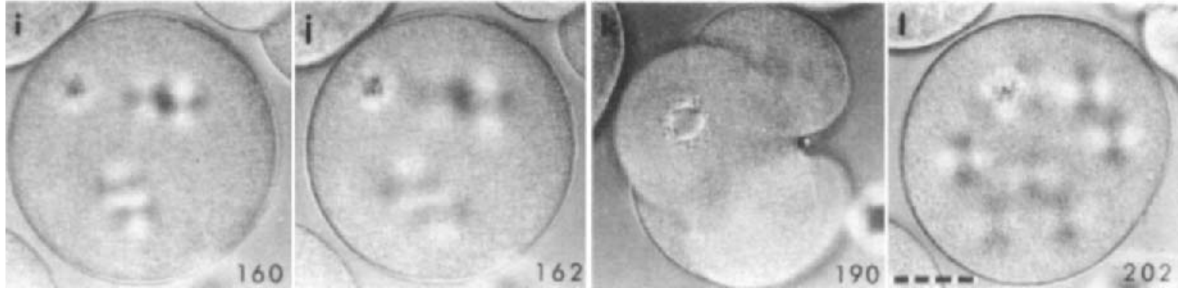

Figure 3. Reproduction of centrosomes in an enucleate zygote. a. Zygote shortly after enucleation in prophase of first mitosis. The single remaining centrosome is centrally located and the oil drop expelled from the micropipette is seen in the upper left portion of the egg. b. First mitosis; the birefringence of the aster increases markedly. c–d. Telophase; the astral focus enlarges and the astral birefringence drops precipitously. e. Second prophase; two weakly birefringent daughter asters are visible. f. Second mitosis; astral birefringence has noticeably increased. g–h. Second telophase; astral birefringence decreases rapidly and the egg initiates a cleavage furrow that later regresses. i–j. Third mitosis; two centrosomes have reproduced to four. k. Third telophase; astral birefringence has faded and multiple furrows are initiated, but later regress. l. Fourth mitosis; four asters have reproduced to eight. Seven asters are visible at this plane of focus. Minutes after fertilization are shown at lower right corner of each frame. 10 μm per scale division.

the number of microtubules nucleated by centrosomes in these enucleated eggs varied cyclically in proper coordination with aster doubling.

To determine if centrioles also reproduced in proper coordination with the doubling of the centrosomal MTOC, we enucleated zygotes and followed them individually until the centrosome had doubled three times to form eight asters. Each zygote was then removed from the preparation, fixed, embedded, and serially 0.25-um sectioned for observation on the high-voltage electron microscope (Sluder et al., 1986). This approach allowed us to know the prior history of each egg examined at the ultrastructural level and to precisely count the centrioles in all eight asters. For all centrosomes serially reconstructed, we found two, and only two, centrioles at the astral focus. Thus, each egg started with two centrioles in the centrosome remaining after the enucleation, and at the time of fixation contained 16 centrioles in eight centrosomes. Thus, centriole duplication and distribution occur in a normal fashion in the absence of nuclear activities. By following three rounds of centrosome reproduction, we could be certain that we were looking at reproduction of centrosomes rather than the subdivision of existing structures as is found when mitosis is prolonged (Sluder and Begg, 1985; Sluder and Rieder, 1985a).

In a separate study, we found that a complete block of DNA synthesis by aphidicolin, a specific inhibitor of the alpha DNA polymerase, does not stop centrosome reproduction in sea urchin zygotes (Sluder and Lewis, 1987). Thus, unreplicated nuclear DNA does not stop centrosome reproduction.

Taken together, these results show that nuclear activities such as transcription, DNA synthesis, or any putative nuclear *signal* at each cell cycle do not control the reproduction of centrosomes. Everything required for centrosome reproduction can be stored as preexisting pools of subunits or as their RNA precursors. Furthermore, the mechanisms that control the number of centrosomes formed and the quantity of subunits recruited from the preexisting pools must be entirely cytoplasmic.

In considering the implications of these results for the control of centrosome reproduction, we must draw a clear distinction between the importance of the execution of particular nuclear events and the importance of the cytoplasmic pathways that drive those nuclear events. Although we have ruled out the possibility that nuclear activities themselves control centrosomal events, we have not addressed the possibility that the mechanisms that drive nuclear events continue to cycle in a

normal fashion and may coordinately drive centrosome reproduction. Studies that bear on this issue will be reviewed below.

We realize that studies on cultured cells, which have shown that enucleation prevents procentriole formation and centrosome reproduction, might question the applicability of studies on egg systems to *typical* somatic cells. In this regard, our key finding is that the minimal, essential mechanisms for centrosome reproduction can be entirely cytoplasmic and independent of the nucleus. The demonstration that cultured cells require a nucleus for centrosome reproduction may only reflect the fact that enucleation stops their cell cycle at a point where the centrosomes are not scheduled to reproduce. Even if specialized interlocks between nuclear activities and the events of centrosome reproduction do exist in cultured cells (see Hartwell and Weinert, 1989), such limits may operate in addition to the minimal cytoplasmic mechanisms that are revealed only in cells that are not growth limited.

Role of Protein Synthesis in Centrosome Reproduction

Protein synthesis is essential for the normal progression of the cell cycle. Not only must cells grow before entering the mitotic cycle, but also passage through interphase and entry into mitosis depend on the synthesis and accumulation of a number of G_1 and G_2 cyclin proteins, which associate with the $P34^{cdc2}$ kinase to form active complexes that phosphorylate stage-specific substrates. In G_2 the cyclin B associates with $P34^{cdc2}$ to form an active complex called maturation or mitosis promoting factor (MPF) (reviewed in Cross et al., 1989; Swenson et al., 1989; Pines and Hunter, 1990; Maller, 1991). As the cyclin accumulates, a series of specific phosphorylations and dephosphorylations of $P34^{cdc2}$ activates its kinase activity and causes subsequent entry into mitosis. Cyclin B abruptly degrades at the metaphase–anaphase transition by proteolysis. This causes MPF activity to precipitously drop, and the cell returns to interphase as mitosis-specific substrates are dephosphorylated (reviewed in Maller, 1991).

In principle, there are two ways in which protein synthesis could play an essential regulatory role in centrosome reproduction. First, the cell could coordinate centrosomal and nuclear events by controlling both by the same $P34^{cdc2}$ pathway. In such a case the importance of protein synthesis would be indirect through the accumulation of G_1 and G_2 cyclins. Second, the cell could control centrosome reproduction by requiring the timed synthesis of one or more subunits necessary for the

assembly of the daughter centrosome. In eggs the translational products could come from the regulated translation of stored maternal messenger RNAs, while in growing cells the availability of these RNAs might be under transcriptional control.

In cultured L929 (mouse) cells at least 4 hours of protein synthesis in late G_1 is required for procentriole formation (Phillips and Rattner, 1976). Given that the inhibition of protein synthesis in cultured cells may block growth, thereby arresting the cell cycle prior to the point when procentriole assembly is scheduled to occur, we investigated this issue in sea urchin zygotes because they do not grow between divisions.

We used a double block to protein synthesis by continuously treating eggs, starting before fertilization with emetine and anisomycin and using concentrations at which either drug alone should be completely effective. These drugs have different targets in the protein synthetic pathway. Anisomycin binds specifically to the 60S subunit of the ribosome, primarily blocking peptide bond formation and, to a lesser extent, inhibiting substrate interaction with the donor and acceptor sites of the peptidyl-transferase center. Emetine selectively prevents EF-2-dependent translocation in polysomes by possibly binding to the 40S subunit of the ribosome (reviewed in Vazquez, 1979).

To empirically determine if this drug combination completely inhibits protein synthesis, we loaded two identical cultures of eggs before fertilization with ^{35}S-methionine—one in the presence and the other in the absence of drugs. At the expected time of first mitosis we processed equal aliquots of zygotes from both cultures for gel electrophoresis on gels that resolve proteins ranging from at least 500 Kd to approximately 5 Kd. Figure 4A shows the electrophoretic pattern of total cell proteins from control and drug-treated zygotes at two loadings. The autoradiograph of this gel is shown in Figure 4B. For the control zygotes there is extensive incorporation of label into newly synthesized proteins (Figure 4B, lanes C_{80} and C_{15}). For the drug treated zygotes there is label incorporation only into material that does not enter the gel and material that runs at the dye front (Figure 4B, lanes D_{80} and D_{15}). No other labeled bands were detectable. The material that does not enter the gels may be glucosaminoglycans that acquire label from the radioactive SO_4 present as a contaminant in the preparations of ^{35}S-methionine used. The label running at the dye front represents unincorporated intracellular ^{35}S-methionine present in these whole cell preparations. Unless awkward assumptions are made concerning the selective synthesis of proteins so large that they do not enter the gel and peptides so small that they run at

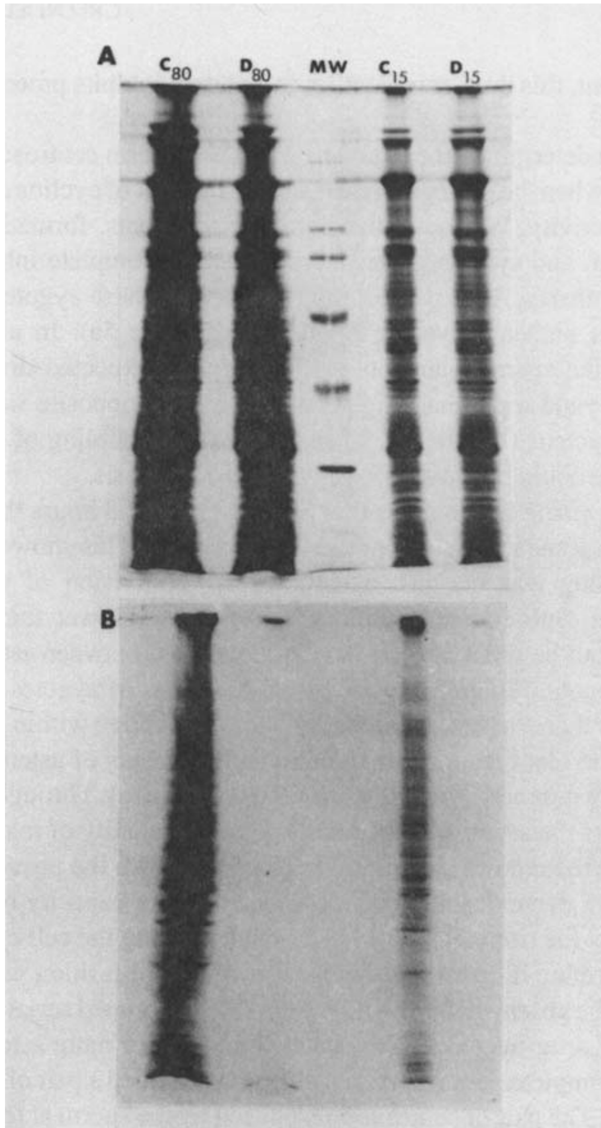

Figure 4. A: SDS-PAGE of total cell protein for control and drug-treated zygotes labeled with ^{35}S-methionine as visualized by Coomassie Brilliant Blue staining. Lanes C_{80} and D_{80}, 80 μl loadings of control and drug-treated eggs respectively. Lane MW: molecular weight markers. Lanes C_{15} and D_{15}, 15 μl loadings of control and drug-treated eggs respectively. B: Autoradiogram of the gel above showing the pattern of ^{35}S incorporation into proteins.

the dye front, this drug combination completely inhibits protein synthesis.

We then determined the extent to which the sperm centrosome could reproduce when the cell cycle is arrested by the lack of cyclins and hence, no MPF activity. We found that fertilization events, formation of the sperm aster, and syngamy were normal despite complete inhibition of protein synthesis. As expected, the cell cycle of these zygotes arrested before first nuclear envelope breakdown (Figure 5a). In all zygotes examined the sperm aster doubled once by the expected time of first mitosis to yield a prophase figure with asters on opposite sides of the enlarged nucleus (Figure 5a). Thus, at least one doubling of the sperm centrosome could occur without any protein synthesis.

The surprising finding was that over a period of 8 hours the number of asters in some zygotes progressively increased. This showed that the first doubling was not just a splitting or subdivision of the sperm centrosome. Subsequent doublings were always slower than normal, asynchronous between zygotes, and asynchronous between asters within a single zygote. Figure 5 shows typical examples of zygotes with 2, 4, and greater than 4 asters. Asynchrony of aster doubling within individual zygotes is evident from cases showing odd numbers of asters or unexpected even numbers, such as 6 and 10 (Figure 5c–e). Throughout these experiments the asters appeared weak, and the quantity of microtubules they nucleated did not cycle. This is consistent with the possibility that microtubule dynamics and centrosome nucleating capacity may be influenced by the rise and fall of MPF activity during the cell cycle.

To determine if centrioles duplicate in a normal fashion when asters double in the absence of protein synthesis, we examined serial semithick sections of drug-treated zygotes that contained 2 or more asters. Of the 33 asters completely reconstructed all but 3 contained a pair of centrioles (Figure 6). The pair of centrioles introduced by the sperm at fertilization must have duplicated and must have been distributed in a normal fashion to daughter centrosomes. Thus, the doubling of asters seen at the light microscope level represents the reproduction of complete centrosomes in the great majority of cases. The finding of 3 asters containing no centrioles is best explained by the occasional splitting of the centrosomal MTOC before the centrioles duplicate. Since sea urchin centrosomes without centrioles do not double between mitoses (Sluder et al., 1989b), the occasional generation of acentriolar asters should lead to the formation of the odd numbers of asters that we observed in some zygotes.

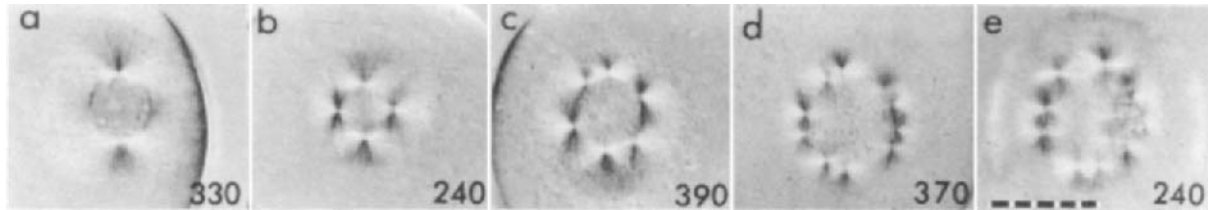

Figure 5. Asters in zygotes in which protein synthesis is completely inhibited. Zygotes are extracted with microtubule-stabilizing buffer and astral birefringence is augmented by hexylene glycol. a. Zygote containing two asters closely associated with the nucleus. b. Zygote containing four asters associated with the nucleus. c. Zygote containing six asters. d. Zygote containing ten asters closely associated with the nucleus. e. Zygote containing multiple asters, eleven of which are visible in this plane of focus. Minutes after fertilization are shown in the lower corner of each frame. 10 μm per scale division.

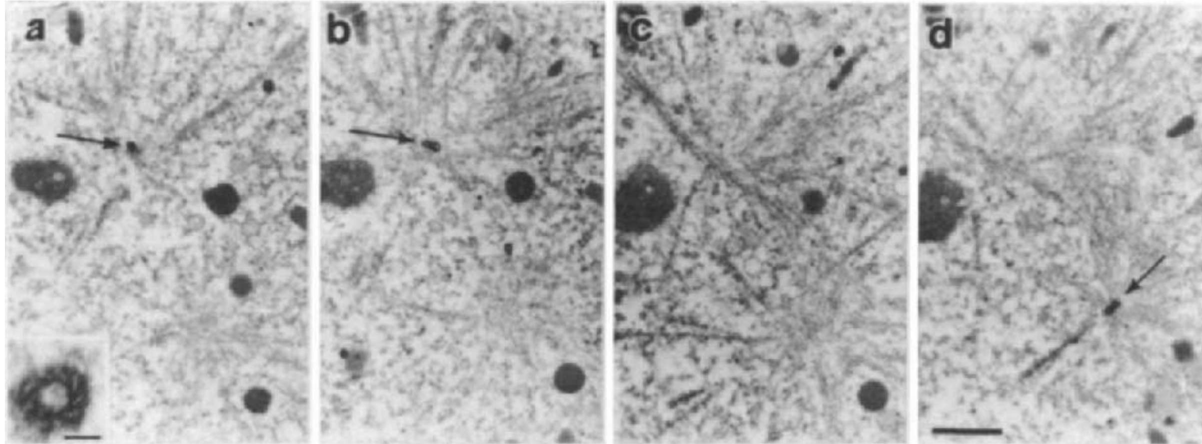

Figure 6. Serial 0.25-μm sections through two closely associated asters in a drug-treated zygote containing multiple asters fixed six hours after fertilization. a. One centriole (arrow) of the upper aster seen in cross section. Inset: higher magnification view of this centriole. b. Both centrioles (arrow) of the upper aster are visible in this section. d. Both centrioles (arrow) of the lower aster are seen in this section. Subsequent sections showed no additional centrioles in either aster. Bars: (a: inset) 0.12 μm, (d) 2 μm.

A concurrently published study on cycloheximide treated *Xenopus* zygotes (Gard et al., 1990) shows that these results are not peculiar to sea urchin zygotes. Their work clearly demonstrates that even though centrosome reproduction may be asynchronous within an embryo, centrosomes always double from 1 to 2 rather than in an unregulated fashion. Furthermore this work indicates that *Xenopus* embryos contain a pool of subunits sufficient to assemble at least 1000 to 2000 complete centrosomes.

These two studies show that cells that are not growth limited do not control the reproduction of centrosomes by requiring the synthesis of one or more structural components of the centrosome at each cell cycle. Even before fertilization the egg contains pools of all the necessary components for the assembly of many centrosomes. Furthermore, the mechanisms that control the recruitment and assembly of these components into centrosomes are not themselves under translational control.

An important finding of these studies is that repeated cycles of centrosome reproduction can proceed even though the cell cycle, as defined by cycles of MPF activity or nuclear events, is arrested in interphase. This indicates that centrosome reproduction and nuclear events must be controlled by different metabolic pathways. Thus, we are left with the intriguing question of how centrosome reproduction is normally tightly coordinated with nuclear events during the cell cycle, even though they can run independently if interphase is greatly prolonged. In egg systems in which centrosomes reproduce when DNA synthesis is initiated in telophase, the precipitous drop in MPF activity could conceivably entrain a pathway that controls the centrosome cycle even though MPF may play no direct role in centrosomal events. However, the observation that the earliest visible events of centrosome reproduction in somatic cells occur at the G_1–S boundary argues that the metaphase–anaphase transition drop in MPF activity is not the only signal and that we must look to other, as yet unknown, mechanisms. This pursuit promises to be difficult since we do not really know when the key initiating events of centrosome reproduction take place; the disorientation of centrioles and the formation of daughter centrioles at the onset of DNA synthesis may conceivably represent the later stages of the reproductive processes.

The report that protein synthesis in late G_1 is required for daughter centriole formation in cultured cells (Phillips and Rattner, 1976) may raise questions about the applicability of studies of egg systems to somatic cells. At a minimum, the synthesis of new proteins at each cell

cycle is not a fundamental strategy used by all cells to control centrosome reproduction. It is, however, possible that the pools of centrosomal components in cultured cells need to be replenished during the cell cycle in which they are used. If so, this limitation operates in addition to the nontranslational control mechanisms for centrosome reproduction.

C. Role of Centrosomes in the Progression of the Cell Cycle

Centrosomes are not simply passive targets of the cell cycle regulatory mechanisms. Below we review studies that suggest the importance of centrosomes in the interphase progression of the cell cycle and studies that show the importance of microtubules nucleated by the centrosomes in the mechanisms that determine when the cell will initiate the metaphase–anaphase transition.

Progression Through Interphase

The work of Maniotis and Schliwa (1991) has led to the proposal that centrosome doubling is a cell cycle checkpoint for entry into mitosis. They microsurgically cut interphase BSC-1 cells between the nucleus and the centrosome to form a karyoplast and a cytoplast containing the centrosome. Between 20 and 30 hours after the operation, the centriole-free karyoplasts reestablish a single astral array of microtubules next to the nucleus and regenerate a Golgi apparatus of normal size. Importantly, the karyoplasts at least initiate (and perhaps complete) DNA synthesis as determined by Brdu incorporation. However, centrioles do not regenerate in the centrosome-like MTOC, and the karyoplasts do not divide into two cells even when followed for as long as 2 weeks, despite growth to abnormally large sizes. In contrast, controls in which one or two pieces of peripheral cytoplasm are amputated divide repeatedly and form colonies within this period.

The clear-cut result of this study is that reassembly of a functional cytoplasmic MTOC is not sufficient for centrioles to reform even though the cells grow and enter S phase, the normal time of daughter centriole formation. This is an important indication that daughter centriole assembly can occur only when a preexisting centriole provides a template or initiation complex, even though adequate cytoplasmic pools of centriolar subunits may be synthesized during the prolonged period of substantial cell growth. Their report that the reassembled MTOC does not double from one to two even over prolonged periods of time indicates that for

cultured cells, like echinoderm eggs, a centrosomal MTOC without centrioles does not have reproductive capacity. Although the failure of the karyoplasts to divide is not surprising given the lack of centrosome doubling, none was observed to enter mitosis as seen by chromosome condensation and nuclear envelope breakdown.

Since cell cycle regulatory proteins, such as $p34^{cdc2}$ (Bailly et al., 1989) and cyclin B (Maldonado-Codina and Glover, 1992), have been localized to the centrosome or the pericentrosomal region, Maniotis and Schliwa (1991) proposed that the karyoplasts do not enter mitosis because removal of centrioles could interfere with the chain of regulatory events that activate the $p34^{cdc2}$ kinase complex. In effect, centrioles, or something spatially associated with them, may mediate the steps that activate this kinase and take the cell into mitosis. More recently Bailly and Bornens (1992) have elaborated on this theme by proposing that the activation of the 34^{cdc2}-cyclin kinase complex depends on the reproduction of the centrosome, presumably by a direct interaction of the kinase with centrosomal components. Perhaps the G_1-S doubling of centrioles, either the assembly of precursor structures or the elaboration of the procentrioles, is the important event. Alternatively, the G_2 division of the complete centrosome into two focal arrays of microtubules that rearranges the cytoskeletal architecture of the cell could somehow influence the activation of MPF. Such possibilities are appealing in that they provide an explanation for how the cell could ensure that it will not enter mitosis until the centrosome has reproduced.

However, there are a number of concerns with these proposals. First, the requirement for a putative centriole-based activation of MPF is not found in all cell types. For example, echinoderm eggs proceed through multiple cell cycles when centrioles are not present and the maternal centrosomal MTOC does not double numerically (Sluder et al., 1989a, 1989b). All higher plant cells and a number of animal cells normally propagate without centrioles (Pickett-Heaps, 1971; Debec et al., 1982; Wheatley, 1982). Also, *Xenopus* egg extracts will go through rounds of the $p34^{cdc2}$ kinase cycle in the absence of centrosomes. Second, the requirement for a rearrangement of the microtubular cytoskeleton prior to entry into mitosis is also not universal. Both sea urchin zygotes and cultured cells will enter mitosis with essentially normal kinetics in the complete absence of cytoplasmic microtubules (Johnson and Rao, 1970; Sluder, 1979). Also, Ts745 Syrian fibroblasts defective in centrosome separation at the nonpermissive temperature clearly do enter mitosis without centrosome separation (Wang et al., 1983).

Thus, the importance of centriole replication and centrosome doubling in the activation of the $p34^{cdc2}$ kinase during interphase is not universal even among somatic cells. Given that Maniotis and Schliwa (1991) did not observe entry into mitosis without centrioles, the proposed centriole–centrosome duplication feedback mechanism deserves further experimental investigation.

Progression Through Mitosis

Spindle microtubules nucleated by the centrosomes are not only involved in the execution of most mitotic events but also play an important role in a feedback or *checkpoint* mechanism that controls the time of the metaphase–anaphase transition that commits the cell to finish mitosis and start the next cell cycle. For all higher eukaryotic cells that have been examined, inhibitors of microtubule assembly delay or arrest the cell cycle in mitosis. Although most cell types examined will eventually show chromatid disjunction and a return to interphase, the total duration of mitosis is always significantly prolonged (Eigsti and Dustin, 1955; Sluder, 1979: Rieder and Palazzo, 1992).

The details of this story are best understood in sea urchin zygotes, a model system well suited to the study of this phenomenon. Zygotes are briefly treated with Colcemid in prophase of first mitosis to prevent microtubule assembly at a dose specific for microtubule assembly (Sluder, 1979). The cell cycle continues as seen by cycles of nuclear envelope breakdown–reformation and regular increases in chromosome number. The duration of each mitosis, however, is almost exactly twice as long and the cell cycle as a whole is correspondingly prolonged (Figure 7). During this prolonged mitosis, the chromosomes in each zygote synchronously split (*c*-anaphase or anaphase onset without chromosome separation) markedly later than anaphase onset in the controls. The timing of this *c*-anaphase relative to nuclear envelope breakdown indicates that essentially all of the extra time these cells spend in mitosis is due to the prolongation of the prometaphase–metaphase portion of mitosis. Once the chromosomes split the reformation of nuclear envelopes around groups of chromosomes and entry into the next cell cycle proceed with normal kinetics. Since the duration of interphase between nuclear envelope reformation and the next nuclear envelope breakdown remains normal, the cell cycle does not speed up to return to its original schedule.

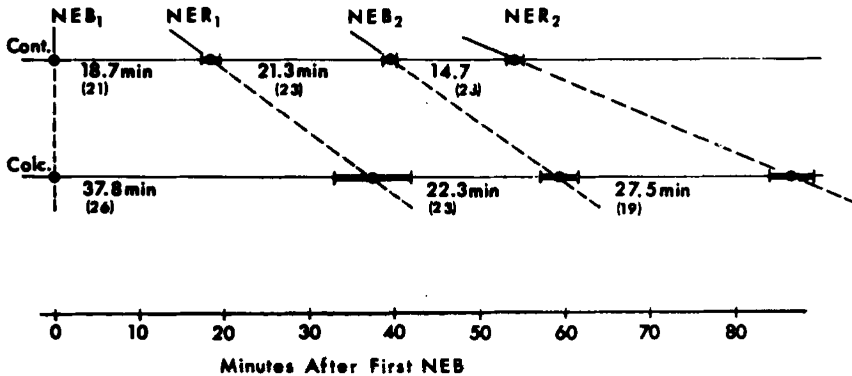

Figure 7. Timing of nuclear envelope breakdown (NEB) and nuclear envelope reformation (NER) in untreated (upper line) and Colcemid treated zygotes (lower line). First NEB is normalized to zero for all individual zygotes. The mean times of NEB and NER are shown as filled circles on the time axes; the heavy horizontal bars represent the 95% confidence limits of the means. The larger numbers under each time axis show the mean duration of the intervals and the smaller numbers give the sample sizes. *L. variegatus* cultured at 22°C.

A separate series of experiments showed that the start of microtubule assembly determines the time of the metaphase–anaphase transition. Zygotes are treated with Colcemid to prevent microtubule assembly and, at various times after nuclear envelope breakdown, individuals are irradiated with 366-nm light to photochemically inactivate the drug and allow microtubule assembly (Aronson and Inoue, 1970; Borisy, 1971; Sluder, 1979). When irradiated at nuclear envelope breakdown, spindle assembly is normal and the zygotes initiate anaphase at the normal time, 10 minutes later. When the irradiation is delayed for times up to the point when the zygotes would have normally been in telophase, the cells still assemble a spindle over the normal time course and initiate anaphase 10 minutes after the irradiation (Figures 8 and 9). Although late in starting, chromosome movement, disassembly of the spindle, splitting of the spindle poles, cleavage, and nuclear envelope reformation proceed at a normal pace properly coordinated with each other. In this fashion prometaphase can be more than doubled in duration, yet mitosis remains normal. The start of the next mitosis at nuclear envelope breakdown is correspondingly delayed relative to the controls. If the start of spindle

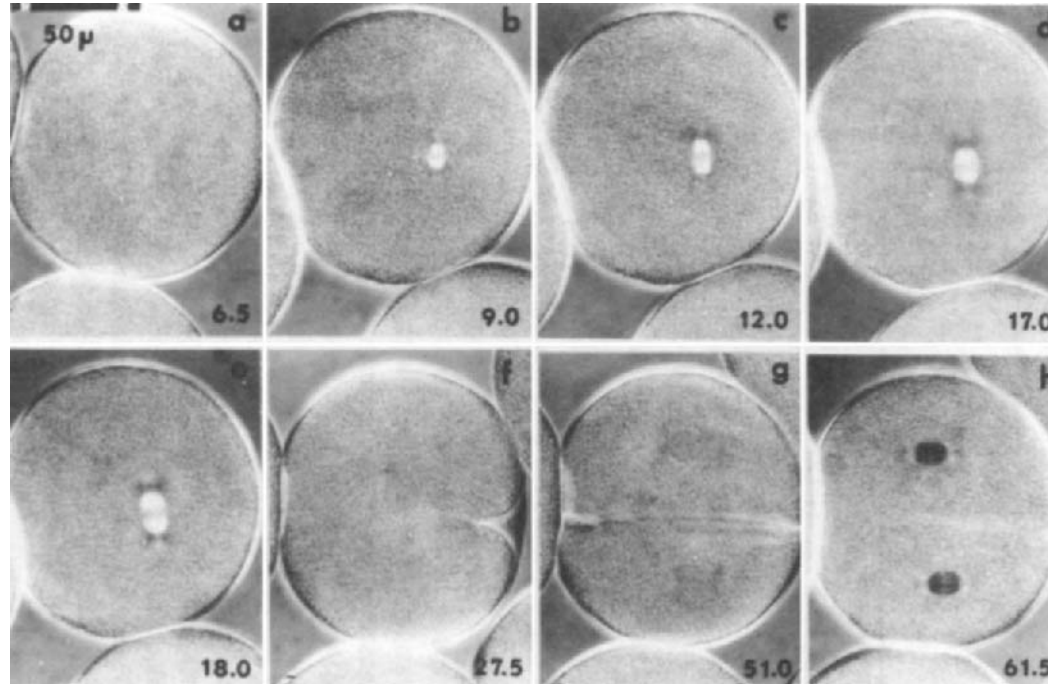

Figure 8. Mitosis in a Colcemid treated zygote after irradiation with 366-nm light 8 minutes after first NEB. a. After NEB but before irradiation. b–d. Spindle assembly with normal kinetics. e. Anaphase onset approximately 10 minutes after the irradiation, which is the normal NEB to anaphase onset interval. f–g. Telophase and reformation of daughter nuclei. h. Second prometaphase is normal. Minutes after first NEB shown in the lower corner of each frame. *L. variegatus* cultured at 22°C. 10 µm per scale division.

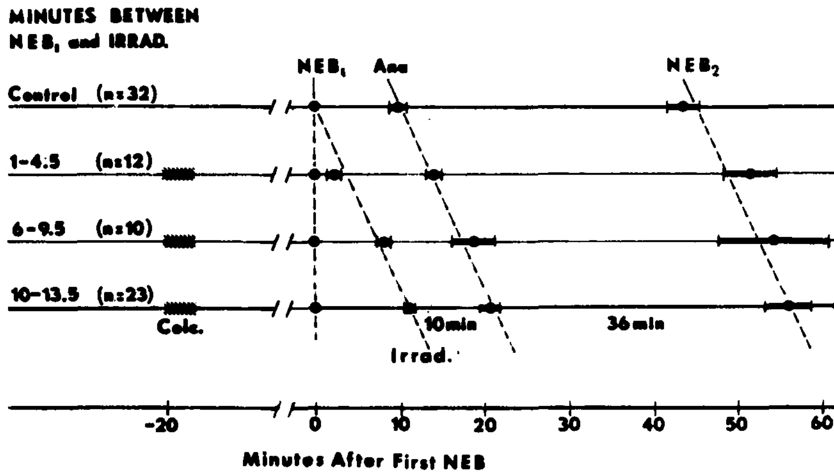

Figure 9. Photochemical inactivation of Colcemid during first mitosis. Top line shows the timing of mitotic events for untreated control zygotes irradiated at NEB. Lower three lines show the timing of mitotic events for Colcemid treated zygotes irradiated at various times after NEB. The data are broken down into three categories based on how long the irradiation was delayed past first NEB (shown in minutes on the far left). First NEB is normalized to zero for all individual zygotes. The mean times of NEB, irradiation, anaphase onset and second NEB are shown as filled circles on the time axes; the heavy horizontal bars represent the 95% confidence limits of the means. The numbers in parentheses on the left give the sample sizes. *L. variegatus* cultured at 22°C.

assembly is delayed significantly past the expected time of telophase, the zygotes show evidence of spontaneously proceeding through mitosis. These results show that inhibition of microtubule assembly does not "arrest cells at metaphase" as has often been said (reviewed in Sluder, 1979; Rieder and Palazzo, 1992); within relatively broad limits, the temporal progression of mitosis does not begin until spindle microtubules start to assemble.

In addition, spindle size influences the timing of mitotic events. Instead of completely blocking spindle microtubule assembly, lower doses of Colcemid diminish the size of spindles and the extent of astral microtubule assembly. In all cases, cells with diminished spindles take longer to go from nuclear envelope breakdown to anaphase onset than their counterparts with normal-size spindles (Figure 10). However, once

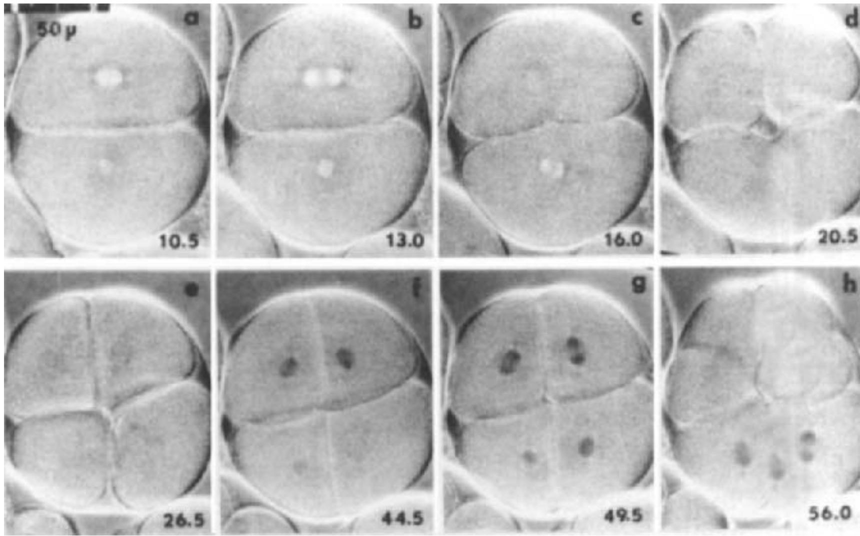

Figure 10. Mitotic events in daughter cells with different size spindles. Zygotes are treated with a low dose of Colcemid to diminish the size of spindles assembled at second mitosis. Second NEB is synchronous in daughter cell pairs. After second NEB one daughter is irradiated to inactivate the Colcemid and allow the cell to assemble a normal-sized spindle to serve as a control against which to compare the timing of the daughter with the smaller spindle. a. Prometaphase after the upper blastomere is irradiated. b–c. Anaphase onset occurs earlier in the daughter with the larger spindle. d–e. Telophase and NER in both cells. f–g. Third NEB occurs earlier in the progeny of the blastomere that entered anaphase first. g–h. Third anaphase onset and cleavage are correspondingly asynchronous. Minutes after second NEB shown in the lower corner of each frame. *L. variegatus* cultured at 22°C. 10 µm per scale division.

the cell initiates the metaphase–anaphase transition, the cell cycle proceeds to the next nuclear envelope breakdown with normal timing regardless of spindle size (Sluder, 1979).

Taken together, these observations indicate that the cell cycle appears to have a fundamental rhythm that allows more time for mitosis than is actually used under normal circumstances. Starting with nuclear envelope breakdown, there is a waiting period that provides the cell with wide

temporal tolerances to assemble the spindle and divide. Within this period, spindle microtubules or some function they perform are part of the mechanism that controls the triggering of the metaphase–anaphase transition with its attendant drop in MPF activity and degradation of cyclin proteins. Once the cell has passed this important transition point, the cell cycle proceeds at a normal pace up to the next nuclear envelope breakdown independent of the presence or absence of microtubules.

Although the assembly of spindle microtubules is clearly important in the timing of mitotic events, the way in which these microtubules are involved in the control mechanisms was not clear. We next investigated the possibility that spindle microtubules act by providing the cell with a structural framework that has the specific geometry for the operation of some necessary process. Thus, we experimentally manipulated the spatial arrangement of spindle microtubules and characterized the changes in the timing of mitotic events (Sluder and Begg, 1983). In early prometaphase of second mitosis, a microneedle is used to cut and separate the asters of one blastomere into two half-spindles (Figure 1l). The unoperated daughter cell serves as a control for comparing the timing of the operated cell. A series of control manipulations showed that the microsurgical operation *per se* does not nonspecifically damage the zygotes. We found that blastomeres with cut spindles take, on average, 48 minutes to proceed from nuclear envelope breakdown to telophase, as opposed to 15 minutes for their same embryo control cells. Once the manipulated cells enter telophase, the time to the start of the next mitosis is normal.

As a second way to produce spindles of altered geometry, we used several methods to prolong mitosis, which indirectly induces the formation of monopolar spindles (Sluder and Begg, 1985). The timing of blastomeres with monopolar spindles was compared to same embryo blastomeres in which two monopoles came together to form a functional bipolar spindle (Figure 1g). Cells with monopolar spindles spent significantly more time in mitosis (49 min on average) than their same embryo controls with bipolar spindles (15 min on average) (Sluder and Begg, 1983).

Taken together, these results are interesting because they show that the timing mechanisms are not dependent upon the total amount of tubulin polymer in the cell or the utilization of the tubulin pool; the arrangement of the microtubules into a normal bipolar configuration is an important facet of the control mechanisms for the metaphase–anaphase transition. The way in which the alterations in the spatial arrange-

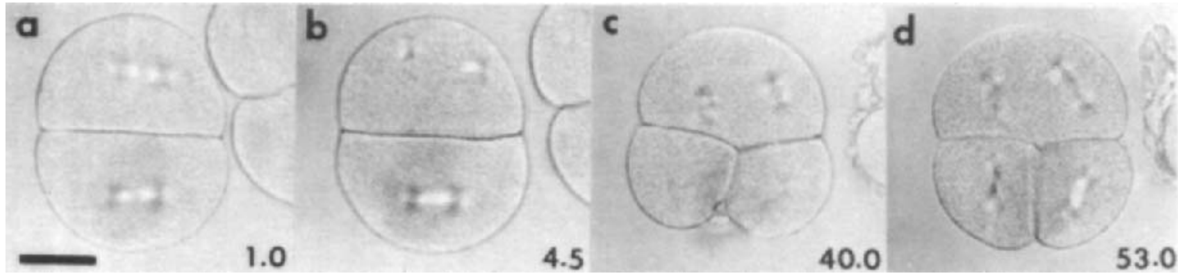

Figure 11. The spatial arrangement of spindle microtubules influences the duration of mitosis. Shortly after the synchronous second NEB in both daughter blastomeres, the spindle of the upper cell is cut with a microneedle. The lower daughter completes mitosis and enters third mitosis with normal kinetics. The upper blastomere remains in mitosis much longer than it normally would have; during the prolonged prometaphase each centrosome splits. Eventually the operated blastomere completes mitosis and enters the next cell cycle. *L. pictus* cultured at 20°C. 10 μm bar.

ment of spindle microtubules prolong mitosis is a mystery. Given that a cell with a small spindle traverses mitosis more slowly than normal, the timing of cells with cut or monopolar spindles could be rationalized by postulating that monopolar spindles have less total polymer than a normal spindle. Also, cutting a spindle might move the asters into functionally separate *universes* in these large cells.

To gain insight into this possibility, we compared the duration of mitosis for zygotes containing Colcemid-diminished bipolar spindles to those containing monopolar spindles or cut spindles (Figure 12). Microtubule distribution and half-spindle birefringence (a measure of microtubule density) indicate that monopolar spindles have at least as much total polymerized tubulin as diminished bipolar spindles. In addition, the asters of the diminished bipolar spindle have markedly shorter and fewer microtubules than the normal sized aster of a monopolar spindle.

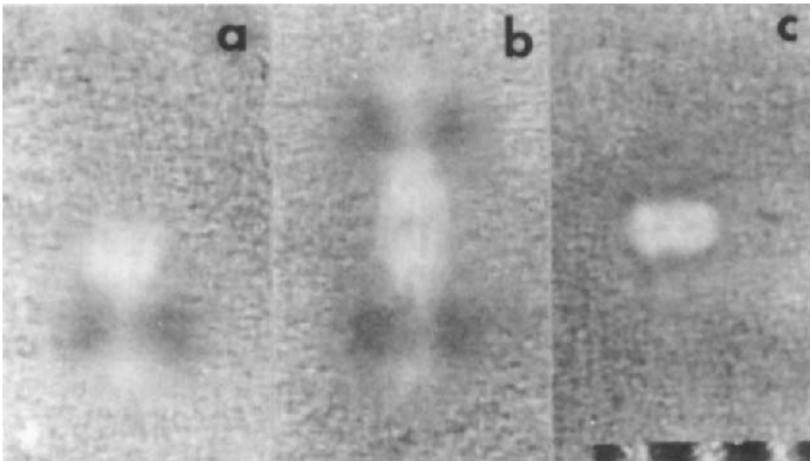

Figure 12. Comparison of (a) monopolar spindle (b) normal spindle (c) Colcemid-diminished bipolar spindle. All photographs printed to the same magnification. The birefringence of the region between the chromosomes and the aster is the same for the monopolar and normal bipolar spindle; aster sizes are the same. The birefringence of the diminished bipolar spindle is approximately half that of the other two but is not faithfully rendered here; asters are so small as to be almost imperceptible. *L. pictus* cultured at 20°C. 10 μm per scale division.

The results of this comparison were surprising. We found that cells with monopolar or cut bipolar spindles spent, on average, more than twice as much time between nuclear envelope breakdown and telophase as cells with diminished bipolar spindles (48 vs. 22 min respectively). Thus, a bipolar spindle, even though much smaller than normal, enables a cell to initiate the metaphase–anaphase transition sooner than a cell containing a spindle with only one pole or two separated poles. The timing mechanisms behave as if they are more sensitive to the bipolarity of the spindle than the total quantity of microtubules assembled. This comparison is important because it rules out simple models for the involvement of spindle microtubules in the timing mechanisms. For example, if astral microtubules are helping to move substances and organelles within the cell, a monopolar spindle with its significantly longer, more plentiful astral microtubules should be more effective than the two almost imperceptible asters of the diminished bipolar spindle.

How then do microtubules nucleated by the centrosomes participate in the mechanisms that control the metaphase–anaphase transition? Although we do not have an answer to this question, we offer two speculations that do little more than suggest where in the cell we might look for an answer. First, the cleavage furrow is triggered at the time of anaphase onset by the interaction of aster pairs with the cell cortex (Rappaport, 1969; 1986). In comparing our results to a number of Rappaport's observations, we note that spindle perturbations that influence the duration of prometaphase are the same as those that influence the extent to which a zygote forms a cleavage furrow. These correlations raise the possibility that an interaction between the asters and the cortex somehow triggers the metaphase–anaphase transition (see Sluder, 1988 for a fuller discussion). If so, the nature of this interaction is unknown.

Second, the trigger for the metaphase–anaphase transition might reside in a kinetochore or centromere activity that is modulated by attachment of chromosomes to spindle microtubules. Based on observations that irradiated newt lung cells seem to *wait* in prometaphase until the last chromosome attaches to the spindle, Zirkle (1970) proposed that unattached kinetochores produce a diffusible inhibitor that prevents or delays entry of the cell into anaphase. A rigorous establishment of this phenomenon has been lacking because of the technical difficulty of quantitating the time from nuclear envelope breakdown to anaphase onset for a sufficient number of cells. Nevertheless, McIntosh (1991) has elaborated on this theme by proposing that centromeres contain stretch-sensitive enzyme(s) that produces this putative inhibitor when the chro-

mosome is not under tension; concurrently, the inhibitor is destroyed at a finite rate by a hypothetical cytoplasmic pathway. When even a single chromosome is not attached to the spindle in a bipolar fashion, the model specifies that its centromere produces enough inhibitor to block the metaphase–anaphase transition. Once the chromosome attaches to both spindle poles, the centromeric enzymes are stretched and inactivated. The cytoplasmic degradative pathway then brings the concentration of inhibitor down below a threshold that allows the metaphase–anaphase transition to occur.

Although this model understandably does not yet have direct experimental confirmation, several observations support it. First, for spermatocytes of some (but not all) mantid species anaphase does not occur and the cells eventually degenerate when a normally paired X chromosome is separate and consequently fails to congress to the spindle equator (Hughes-Schrader, 1948; Nicklas and Arana, 1992). Second, Bernat et al. (1990), working with HeLa cells, found that injection during G_2 of affinity purified autoimmune sera against the CENP-B centromeric protein allows seemingly normal spindle assembly but blocks the cell cycle in mitosis (also discussed in Earnshaw et al., 1991). Third, Spencer and Hieter (1992) introduced into yeast a supernumerary chromosome that is not necessary for cell viability. They found that mutational or deletional manipulation of the centromeric DNA sequence of this extra chromosome led to a prolongation of mitosis that was independent of the rate at which this supernumerary chromosome was lost. The results of these studies are consistent with the possibility that partial disruption of centromeric structure of one or more chromosomes prevents a normal attachment of the kinetochores to the spindle and the consequent production of a putative inhibitor that blocks the metaphase–anaphase transition. Given that a straight line can be drawn between two points, the observations on HeLa and yeast cells suggest that this proposed feedback control mechanism for the time of anaphase onset may be applicable "from yeast to man."

Since the time of the metaphase–anaphase transition in sea urchin zygotes is influenced by spindle microtubule assembly and function, we sought to directly test the extent to which unattached chromosomes delay anaphase onset in this experimental system. To do so, we compared the time from nuclear envelope breakdown (NEB) to anaphase onset for zygotes having 50% of the chromosomes not attached to the spindle against control zygotes with all chromosomes attached (Sluder and Miller, 1992). Eggs are treated with Colcemid before fertilization to

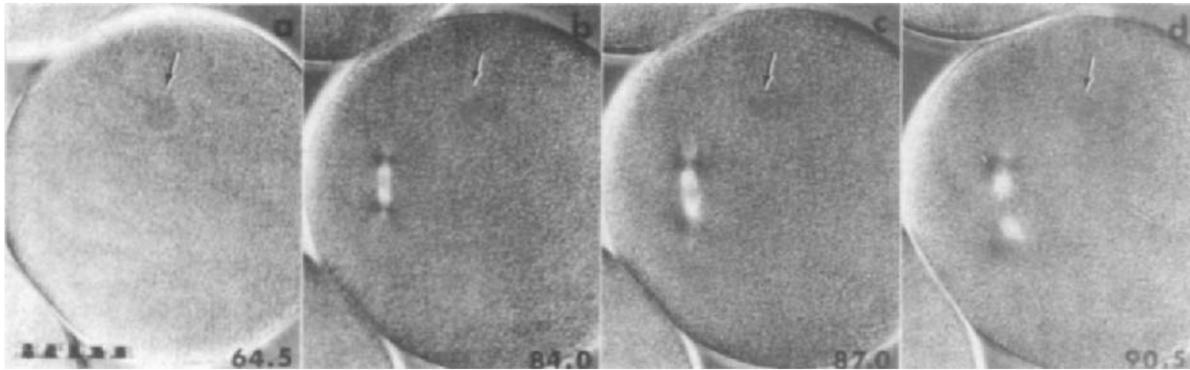

Figure 13. Development of a zygote with separate pronuclei. Zygotes are treated with Colcemid before fertilization to prevent future microtubule assembly and thus, pronuclear fusion. After first NEB individual zygotes are irradiated with 366-nm light to photochemically inactivate the drug and allow microtubule assembly. a. After first NEB but before irradiation. The smaller male pronuclear area is shown by arrowhead and the female pronuclear area by the arrow. b–c. A functional bipolar spindle of normal appearance is assembled in association with the male chromosomes. The female chromosomes lie in a small hyaline area and do not assemble microtubules. d. Anaphase onset for the male spindle. Minutes after fertilization shown in the corner of each frame. *L. variegatus* cultured at 22°C. 10 μm per scale division.

Table 1.

Minutes from Nuclear Envelope Breakdown to Anaphase Onset			
	Average	*n*	*s*
100% of chromosomes attached to spindle:	19.3	51	4.6
50% of chromosomes attached to spindle:	20.5	47	5.9

Minutes from Nuclear Envelope Breakdown to Nuclear Envelope Reformation			
	Average	*n*	*s*
Colcemid block to microtubule assembly:	59.8	29	14.8
lumicolchicine controls:	23.0	28	4.8

Notes: All values in minutes. *Lumicolchicine controls* are zygotes treated with lumicolchicine (the inactive isomer of colchicine that has the same side effects as the parent drug) at the same concentration as the native Colcemid to control for nonspecific effects of the native drug. "n" denotes the number of zygotes scored and "s" denotes the standard deviation. All data are for *L. pictus* cultured at 18°C.

block assembly of the sperm aster, and hence, pronuclear fusion. After NEB of both nuclei, zygotes are irradiated with 366-nm light to inactivate the drug and allow spindle assembly (Figure 13). As previously shown by correlative light and serial section electron microscopy (Sluder and Rieder, 1985b), a functional bipolar spindle is assembled in association with the paternal chromosomes while the condensed maternal chromosomes remain in a small hyaline area unattached to any microtubules. Controls are similarly treated with Colcemid *after syngamy* and irradiated after NEB.

We found that the time from NEB to anaphase onset is the same in control and experimental cells, even when the unattached maternal chromosomes are in the vicinity of the spindle (Table 1). This is not the maximum amount of time that these zygotes will spend in mitosis before spontaneously proceeding into interphase. When Colcemid blocks microtubule assembly, these zygotes spend approximately 59 minutes in mitosis (Table 1). At face value, these results indicate that unattached chromosomes do not delay the metaphase–anaphase transition in this system.

However, we needed to address the concern that the unattached maternal chromosomes might be far enough from the paternal spindle that functionally they would be in their own "world," and thus not be able to exert an inhibitory influence on the spindle. To address this issue, we fluorescently labeled chromosomes of zygotes in which the pronuclei

were separate and used low light level video methods to determine when the unattached chromosomes split (anaphase onset without chromosome movement) relative to anaphase onset for the spindle. Should the unattached maternal chromosomes be truly in their own world, the time of their disjunction would be independent of anaphase onset for the spindle.

We found that the unattached chromosomes disjoined within a few minutes of anaphase onset for the spindle in the same cell. Since the disjunction of the unattached maternal chromosomes is correlated with anaphase onset for the spindle, not the time of the breakdown of the maternal nucleus, the unattached chromosomes do not behave as if they are operating independently of the sperm spindle. Thus, kinetochore attachment to the spindle does not appear to be a negative feedback element in the mechanism that controls the metaphase–anaphase transition in sea urchin zygotes. If the unattached kinetochores (or centromeres) produced a putative inhibitor, it would have to be so weak that fully half the chromosomes (approximately 20) cannot block the metaphase–anaphase transition, or the putative inhibitor would have to be essentially non-diffusible. In either case, the negative feedback mechanism would be functionally ineffective.

That being the case, we are still left with the mystery of how centrosome nucleated spindle microtubules play a role in the mechanisms that control the time of the metaphase–anaphase transition. A new and exciting approach to this problem has been the isolation of six mutants in yeast that do not arrest at mitosis when microtubule assembly is inhibited (Hoyt et al., 1991; Li and Murray, 1991). At the moment all we know about these mutants is that one of these genes appears to code for a calcium binding protein (Li and Murray, 1991).

III. CONCLUDING REMARKS

The thesis of this review has been that the interrelationship between centrosomes and the cell cycle is multifaceted. Some centrosomal activities, such as microtubule nucleation, appear to be driven by changes in cytoplasmic conditions attendant with entry and exit from mitosis. Other activities such as the splitting and duplication events of centrosome reproduction have a more complicated and less well understood relationship with the cell cycle. Splitting, normally a telophase or interphase event, can occur once during a prolonged prometaphase, yet the duplication event is seen only to occur once the cell has passed the metaphase–anaphase transition point. In addition, the complete cycle of centrosome

reproduction can occur multiple times when the cell is held in interphase by a complete block to the synthesis of the cyclin proteins. That centrosome reproduction is not driven in any absolute sense by the MPF pathway begs the question of what mechanism normally coordinates centrosome duplication with nuclear events during the cell cycle. To this question we presently have no answer.

In addition, centrosomes may have an important role in driving the cell cycle. Maniotis and Schliwa (1991) and Bailly and Bornens (1992) propose that centrosomes have an integral role in the activation of MPF during interphase that takes the cell into mitosis. Given the evidence used to support this model, it is presently an intriguing possibility that would benefit from further study. At a minimum, the observation that BSC cells do not enter mitosis when deprived of their centrioles may point to differences in how eggs and somatic cells regulate their cell cycle.

The role of centrosomes in the mechanisms that determine when a cell will go through the metaphase–anaphase transition point is indirect, not direct. The effects of spindle perturbations on the time of anaphase onset are due to the microtubules nucleated by the centrosomes and are not a function of centrosome number. This is most clearly demonstrated in the experiments in which spindle microtubules are prevented from assembling and in which the spindle is cut. In both cases the cell has a normal complement of centrosomes, yet the duration of mitosis is markedly prolonged. The way in which spindle microtubules participate in the control mechanisms for the metaphase–anaphase transition is a mystery, and I have reviewed observations that suggest interesting avenues for further investigation.

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REGULATION OF CENTROSOME FUNCTION DURING MITOSIS

Brigitte Buendia and Eric Karsenti

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ABSTRACT

In this chapter, we focus on the regulation of the microtubule-nucleating activity of centrosomes in mitosis. Progression through the different phases of the cell cycle appears to be driven by the successive activation of different cyclin-dependent kinases. We first review the present evidence indicating that the nucleating activity of centrosomes in animal cells is increased in mitosis by a cyclin-dependent kinase phosphorylation event that affects the capacity of centrosomes to nucleate microtubules. The growing evidence that microtubule nucleation at centrosomes and microtubule dynamics are regulated independently by different cyclin-dependent kinases and specific molecules is discussed. Several molecules are found to be localized at the centrosome *in vivo* including cyclins and different kinases as the cdc2 kinase. We discuss the functional meaning of the localization of cyclins to the centrosomes, emphasizing the possibility that centrosomal localization may not reflect a direct function in the regulation of microtubule nucleation. We still know little about the organization of the microtubule-nucleating material in the centrosome. It is therefore difficult to dwell on the molecular mechanism that could regulate microtubule nucleation during mitosis. We discuss the various possibilities, knowing that γ-tubulin is probably a key protein in microtubule nucleation. We think that regulation of microtubule nucleation by phosphorylation could work through a modulation of the affinity of microtubule-nucleating material for the centriole, a structural modification of the pericentriolar material, or both. Finally, we discuss recent functional assays used to fractionate microtubule-nucleating material from egg extracts and the problems associated with this approach. The increased nucleating activity of centrosomes during mitosis is probably important to facilitate interaction between dynamic microtubules and targets that specify spindle orientation in prophase or kinetochores in metaphase.

In eukaryotic cells, microtubule assembly is nucleated in defined regions of the cytoplasm by specialized structures called microtubule organizing centers (MTOCs). MTOCs can assume various shapes, but in dividing animal cells microtubules are nucleated by centrosomes. We have previously defined the centrosome as a pair of centrioles surrounded by the fibrogranular pericentriolar material (PCM) that nucleates microtubules (Karsenti, 1991). The centrosome defined in this way belongs to the general class of MTOCs; however, centrioles devoid of PCM or PCM devoid of centrioles are not centrosomes. This definition is at odds with that of Mazia (Kalt and Schliwa, 1993; Mazia, 1984) and is clearly discussed by Bornens (Bornens, 1992). We prefer our definition because

of its operational simplicity; it allows us to call different structures by their names. For example, in polarized epithelial cells (MDCK), the MTOC is localized in the apical domain from which microtubules grow toward the base of the cells (Bacallao et al., 1989). Apparently, the centrioles do not nucleate microtubules. Therefore, these cells do not contain a proper centrosome. However, when they enter prophase, they acquire two centrosomes that nucleate microtubules, probably because the centrioles become surrounded by an MTOC (Bré et al., 1990, Figure 1). Whether the MTOC present in the apical domain of the interphasic cells and the MTOC present around centrioles in prophase are entirely similar is unknown. Their protein composition may be entirely identical, in which case the interaction of MTOC material with centrioles could be regulated by posttranslational modifications. Alternatively, the mitotic and interphasic MTOCs may share some proteins or have radically different compositions. The proteins involved in microtubule nucleation are likely to be very similar in both structures.

This particular example raises several questions: (1) what is the nature of the proteins that compose MTOCs? (2) how is the interaction between MTOC proteins and the centrioles regulated? and (3) how is the microtubule nucleating activity of MTOCs regulated? The microtubule-nucleating activity of MTOCs endows them with several functions, including orientation and organization of microtubule networks and orientation of spindle axis during mitosis. The centrosome may also have other still-hidden functions, such as regulation of the cell cycle (Maniotis and Schliwa, 1991; Picard et al., 1987).

Presently, the most tractable question deals with the regulation of microtubule nucleation by MTOcs during the cell cycle and cell differentiation. In this review, we will discuss recent results dealing with the regulation of the function of the centrosome in animal cells during the onset of mitosis. We will not address the question of centrosome duplication (see Johnson and Rosenbaum, 1992; Karsenti, 1991; Rose et al., 1993), but rather focus on the regulation of the microtubule-nucleating activity of centrosomes and why this may be important for cell division and mitotic spindle assembly. Several reviews have been published recently on the rapidly growing family of proteins that belong to the centrosome (Bornens, 1992; Fuller et al., 1992; Kalt and Schliwa, 1993; Kuriyama, 1992; Oakley, 1992).

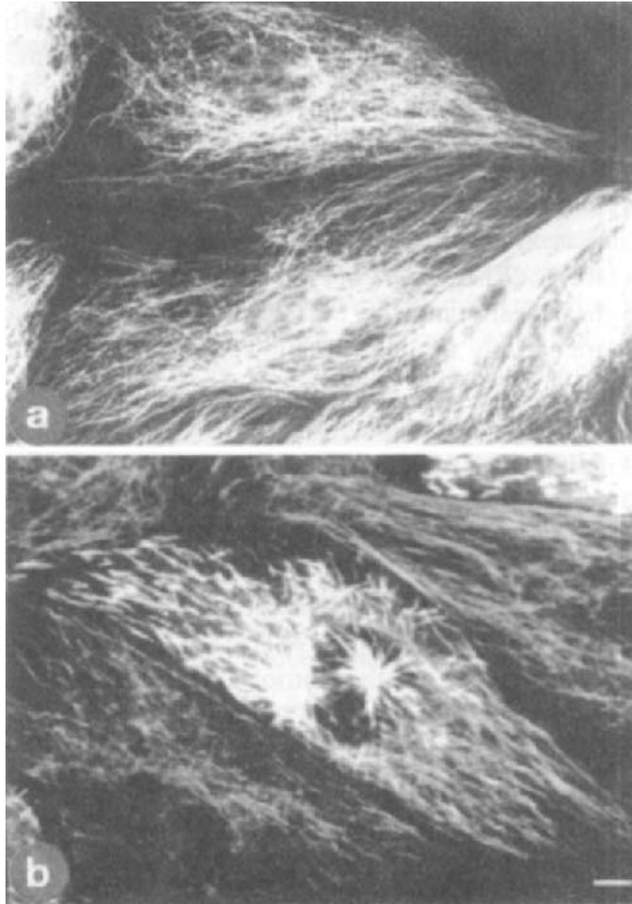


Figure 1. In epithelial cells (MDCK), centrosomes start to nucleate microtubules in early prophase. MDCK cells grown on glass coverslips were fixed and the microtubules revealed by staining with antitubulin antibodies and secondary antibodies labeled with rhodamine (see Bré et al., 1990). (a) in interphase; these cells do not contain an obvious center for microtubule nucleation. Electron microscopy analysis shows that the centrioles have little pericentriolar material and nucleate almost no microtubules (Bré et al., 1987). (b) In prophase, the centrosomes nucleate many microtubules while the interphase network just begins to fragment. In this cell, the nucleus was still intact indicating that it was in early prophase.

I. REGULATION OF THE MICROTUBULE NUCLEATING ACTIVITY OF CENTROSOMES BY CYCLIN DEPENDENT KINASES

In early embryos, fibroblasts, and most highly motile cells, the microtubule network radiates from one centrosome during interphase. At the onset of prophase, after centrosome duplication during S-phase, the two new centrosomes start to migrate around the nucleus, each nucleating a large microtubule aster while the interphasic network disappears. It is at this time in the cell cycle that the nucleating activity of centrosomes starts to increase (Figure 1). This change could be due either to a modification of microtubule dynamics or to a change in the intrinsic nucleating activity of centrosomes. Indeed, it seems that the steady-state number of microtubules nucleated by isolated centrosomes incubated in pure tubulin solutions depends on the dynamic properties of microtubules (Bré and Karsenti, 1990). But we also know that mitotic centrosomes incubated in pure tubulin nucleate more microtubules than interphasic centrosomes (reviewed in Kuriyama, 1992). This raises two interesting questions: (1) which cell cycle signal(s) induce(s) the concerted changes in the microtubule-nucleating activity of centrosomes and microtubule dynamics? and (2) how do the signals modulate the intrinsic nucleating activity of centrosomes in concert with microtubule dynamics?

It is now clear that the successive phases of the cell cycle are driven by the periodic activation of specific kinases composed of one catalytic subunit, the cdc2 kinase or related proteins, and one regulatory subunit belonging to a class of molecules called cyclins. Different cyclins accumulate during G1, S, and M-phase and govern the activity of various cellular compartments through interaction with other molecules or specific kinases (Bischoff et al., 1990; Dirick and Nasmyth, 1991; Fang and Newport, 1991; Futcher, 1991; Hunt, 1991; Hunter and Pines, 1991; Nasmyth and Dirick, 1991; Ogas et al., 1991; Reed, 1991). At the onset of S-phase, at least two different types of cyclin molecules accumulate, called A and B types. The A-type cyclin associates with $p33^{cdck2}$ and $p34^{cdc2}$ kinase subunits leading to their activation (Clarke et al., 1992; Pagano et al., 1992; Solomon et al., 1990). During S-phase cyclin A is exclusively nuclear. It becomes partially cytoplasmic and associates with the centrosomes in prophase. Cyclin B is cytoplasmic and associated with centrosomes as soon as it starts to accumulate and becomes partially nuclear in prometaphase (Bailly et al., 1992; Gallant and Nigg, 1992; Pagano, et al., 1992; Pines and Hunter, 1991).

Previous work had indicated that the microtubule-nucleating activity of centrosomes could be governed by phosphorylation. Indeed, a correlation was established between the increased microtubule-nucleating activity of centrosomes in mitosis and their phosphorylation level as revealed by an increased reactivity to antibodies raised against mitotic phosphoproteins (MPM2 antibody). These antibodies also block the nucleating activity of centrosomes (Centonze and Borisy, 1990; Nislow et al., 1990). Although it was likely that this phosphorylation was dependent upon the activity of cyclin-dependent kinases, it was not easy to determine *in vivo*. Also, the nature of the cyclin-dependent kinase was hard to determine in the living cell. Indeed, systems in which one can use genetics are often not very good for visual observation of the cytoskeleton, and cyclin genes are often redundant making it difficult to examine the function of a single gene in mutants. In addition, different cyclin molecules have partially overlapping functions when overexpressed in cells. Finally, the regulation of the intrinsic microtubule-nucleating activity of centrosomes has to be examined independently from changes in microtubule dynamics.

We decided to examine this point *in vitro*. The use of concentrated *Xenopus* egg extracts was appealing because this is a semi *in vitro* system in which the *in vivo* cytoplasmic conditions are well preserved. One can believe this because mitotic spindles or nuclei can assemble in these extracts (Lohka and Maller, 1985; Lohka and Masui, 1983); the protein phosphorylation pattern in interphasic and mitotic extracts is very similar to that found *in vivo* (Félix et al., 1989); and the early embryonic *Xenopus* egg cell cycle occurs in such extracts (Félix, et al., 1989; Hutchison et al., 1988; Murray and Kirschner, 1989). Moreover, extracts can be prepared in an interphasic state containing large amounts of cyclin-dependent kinase catalytic subunits without cyclin.

The kinase can then be activated by addition to the extract of defined cyclin subunits produced in bacteria. Once in the extract the cyclin binds to the kinase subunit and activates it. Under these conditions, both cyclin A and cyclin B bind almost exclusively to $p34^{cdc2}$ (Figure 2, see Clarke, et al., 1992; Solomon et al., 1990). To examine the role of cyclin-dependent kinases in microtubule nucleation in this system, we used centrosomes isolated from human lymphoid cells (Bornens et al., 1987) reversibly inactivated by a mild urea treatment (Figure 3; Klotz et al., 1990). The centrosomes were then incubated in interphasic extracts or in extracts in which the cdc2 kinase was activated by adding cyclin A or cyclin B, reisolated on a sucrose gradient, and their microtubule-nucle-

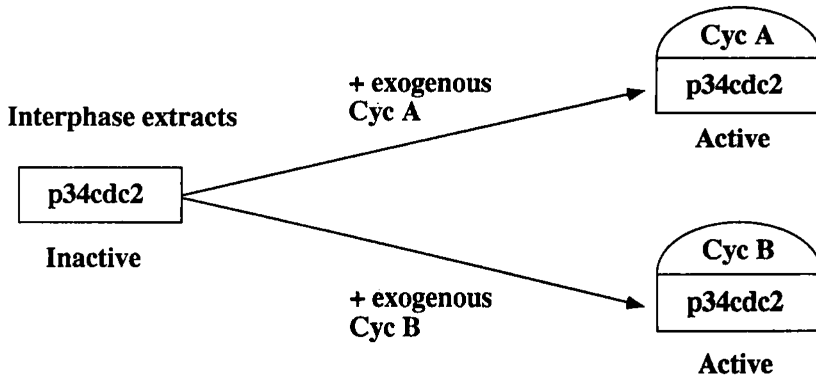


Figure 2. Activation of cdc2 kinase by exogenous cyclins in *Xenopus* egg extracts. Concentrated extracts prepared form *Xenopus* eggs arrested in interphase (Félix et al., 1993), contain a store of free inactive $p34^{cdc2}$ subunits. Cyclins produced in bacteria and added to the extract bind to the endogenous catalytic subunits, activate it, and target it to specific functions.

ating activity assayed by incubation in various concentrations of purified bovine brain tubulin (Buendia et al., 1992). In principle, this approach has several advantages. This is a functional complementation assay that can be used to purify at least part of the material involved in microtubule nucleation. How this material is stored in *Xenopus* eggs can be determined at the same time. Finally, one can study the regulation of the microtubule-nucleating activity by cytoplasmic factors independent of the dynamic state of microtubules in the cytoplasm since microtubule-nucleating activity is assayed in pure solutions of tubulin after reisolation of the centrosomes.

We found that centrosomes incubated in interphasic extracts could nucleate microtubules at a lower tubulin concentration than the native centrosomes, suggesting that the material stored in the eggs has a higher affinity for tubulin than that present on the human centrosomes. In addition, the cyclin-B-dependent kinase did not increase dramatically the nucleating activity of centrosomes, whereas the cyclin-A-dependent kinase was much more efficient. Most interestingly, this kinase increased the average number of microtubules nucleated per centrosome at high tubulin concentrations but did not reduce the critical concentration for nucleation (Buendia, et al., 1992). This result suggests that phosphorylation by a cyclin-A-dependent kinase affects the organization of the

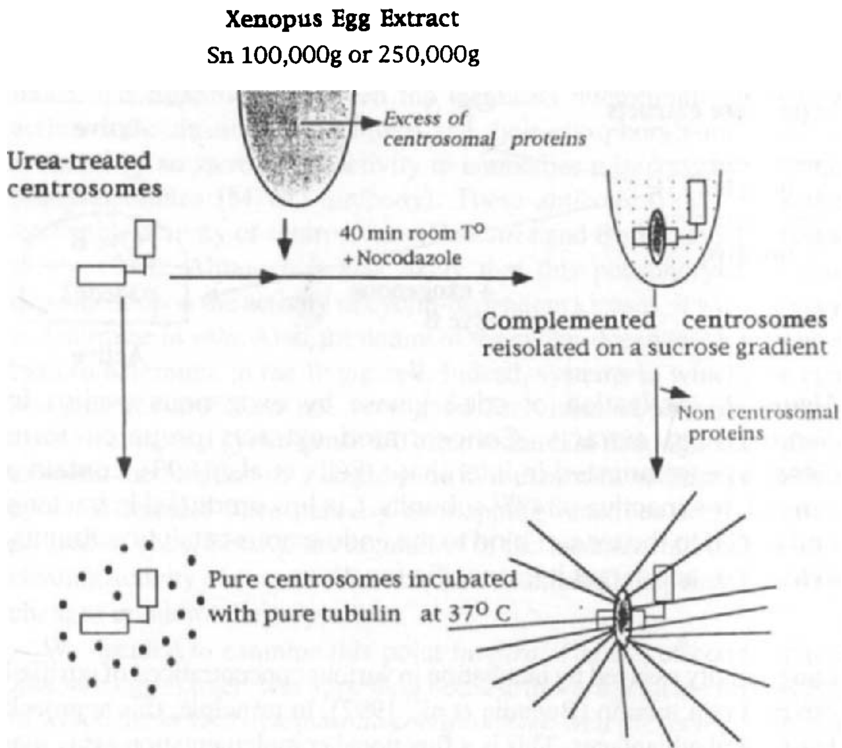


Figure 3. Functional complementation of centrosomes in *Xenopus* egg extracts. Centrosomes isolated from human lymphocytes (Bornens, et al., 1987) retain their capacity to nucleate microtubules when incubated in pure tubulin solutions. After a mild treatment with 2 M urea, they loose this capacity (left part of the figure). However, after incubation in an egg extract and reisolation on a sucrose gradient, they recover the capacity to nucleate microtubules in pure tubulin solutions (right part of the figure). Centrioles are represented by rectangles and the pericentriolar material by a shaded oval; tubulin subunits by dots; and microtubules by lines.

nucleating material in such a way that the capacity of centrosomes to nucleate microtubules is increased. We will come back to the meaning of this observation. The finding that the nucleating activity of centrosomes can be increased by a cyclin-dependent kinase agrees with previous results showing that the nucleating activity of centrosomes is

increased during mitosis in a phosphorylation-dependent manner (Centonze and Borisy, 1990; Kuriyama, 1989; Kuriyama and Maekawa, 1992; Wickramasinghe and Albertini, 1992). Also, Masuda et al. (1992) found that interphasic yeast spindle pole bodies could be induced to nucleate microtubules by incubation in egg extracts containing active cyclin-B-dependent kinase but not by incubation in interphase extracts. Although we do not find an effect of cyclin-B-dependent kinase on the nucleating activity of animal centrosomes, Masuda's result is not really comparable to our studies because in yeast, there is no cyclin A, and the regulation of spindle pole bodies may be different since it involves the induction of new nucleation towards the nucleus for spindle assembly and not an overall increase in microtubule-nucleating activity.

The finding that a cyclin-A-dependent kinase increases the nucleating activity of centrosomes (Buendia, et al., 1992), while it has little effect on microtubule dynamics; that cyclin-B-dependent kinase has the opposite effect (Belmont et al., 1990; Verde et al., 1992); and that the cyclin-A-dependent kinase is activated before the cyclin-B-dependent kinase (Clarke, et al., 1992; Minshull et al., 1990; Pines and Hunter, 1990) supports the hypothesis that a cascade of cyclin-dependent kinases with specific temporal functions is operative in the cell cycle.

II. FUNCTIONAL MEANING OF CYCLINS LOCALIZATION AT THE CENTROSOME

We were first surprised by the lack of effect of the cyclin-B-dependent kinase on the microtubule-nucleating activity of centrosomes. Indeed, the global phosphorylation level of centrosomes as detected by staining with the MPM2 antibody is increased by purified cdc2 kinase (Verde et al., 1990) as well as by the endogenous cdc2 activated by cyclin B added to the extract (B. Buendia, unpublished). The lack of concomitant increase in the microtubule-nucleating activity of these centrosomes suggests that the sites phosphorylated by the cyclin-B-dependent kinase are not involved in the regulation of the nucleating activity of centrosomes. *In vivo*, centrosomes are strongly labeled by cyclin-B antibodies whereas cyclin-A antibodies mostly stain nuclei. However, careful immunofluorescence observations do reveal a centrosomal staining with cyclin-A antibodies in prophase when the cyclin-A-dependent kinase is active and the nucleating activity of centrosomes increased (Bailly et al., 1992; Pagano et al., 1992). Moreover, the timing of activation of the cyclin-B-dependent kinase does not correlate with the timing of in-

creased microtubule nucleation of centrosomes. Therefore, it is likely that in animal cells the nucleating activity of centrosomes is regulated by a cyclin A or related cyclin-dependent kinase, although more data will be required to be certain.

If the localization of cyclin B to centrosomes does not reflect a function in the regulation of microtubule nucleation, then what could it mean? There is one very interesting interpretation of this result. The centrosomal localization of the cyclin B–cdc2 complex may be required to produce a local concentration of active cyclin-B-dependent kinase at the poles of the spindle, and this may have an important function in regulating microtubule dynamics during mitosis. Indeed, we have proposed that microtubule steady-state length is governed by the frequency with which microtubules transit from the growing to the shrinking phase (frequency of catastrophes, f_{cat}) and that the value of f_{cat} could be governed by the concentration of a factor most active in its phosphorylated form (Karsenti, 1991; Verde et al., 1992; Verde et al., 1990). In this model the concentration of phosphorylated factor at any point in the cell would depend on relative rates of phosphorylation and dephosphorylation reactions. If the cyclin-B-dependent kinase is localized on centrosomes and the phosphatase that opposes it is localized on chromosomes, a gradient of phosphorylated factor could be established between the poles and the equator of the spindle, allowing microtubules to grow preferentially towards the chromosomes (Fernandez et al., 1992; Karsenti et al., 1991). This may also have important implications for the localization of chromosomes on the metaphase plate since there are motors in the kinetochores with directional control regulated by phosphorylation (Hyman and Mitchison, 1991a; Hyman and Mitchison, 1991b; Hyman and Stearns, 1992). These ideas are still quite speculative and can be tested, and this shows that the mere localization of a cyclin at the centrosome does not necessarily imply a function in the regulation of the activity of the centrosome itself. According to the hypothesis, the centrosome is an organelle capable of concentrating enzymes in one precise location in the cell, although the effect of the enzyme could be on soluble proteins that diffuse away and carry out their function at a distance from the point where they are modified. We believe that this view of localized enzyme function has to be further developed and tested; it may subsequently prove to be an important mechanism in cellular morphogenesis.

III. HOW DO CYCLIN-DEPENDENT KINASES AFFECT PROTEINS INVOLVED IN THE REGULATION OF MICROTUBULE NUCLEATION?

In animal cells, the increased microtubule-nucleating activity of centrosomes in prophase is apparently due to a phosphorylation event induced by the cyclin-A-dependent kinase or another cyclin-dependent kinase similar to cyclin A. However, we do not know if this kinase phosphorylates proteins involved in microtubule nucleation directly or through a cascade of other kinases, and we do not know whether phosphorylation affects only the structure of the MTOC or the recruitment of proteins stored in the cytoplasm and required for nucleation.

Several proteins have been shown to localize at the centrosome in prophase. Some originate from the nucleus, others from the cytoplasm, and still others are present at the centrosome throughout the cell cycle, but their concentration at the centrosome increases in prophase. This subject is reviewed very clearly by Kalt and Schliwa (Kalt and Schliwa, 1993). Some of these proteins may be directly involved in microtubule nucleation like γ-tubulin (Oakley, 1992), others may have a *linker* function, allowing proteins involved in microtubule nucleation to bind to the pericentriolar region, and others may have nothing to do with microtubule nucleation. At present, it is difficult to understand how the nucleating activity of centrosomes is regulated by phosphorylation. Indeed, this could be through the regulation of the affinity of linker proteins for the centrosome and/or for proteins involved in microtubule nucleation. The spatial organization of proteins like γ-tubulin may also be regulated by phosphorylation.

In a previous paper, we suggested that centrosomal proteins could be recruited by transport along microtubules in a dynein-dependent way (Verde et al., 1991). This may be the case for some proteins (see a similar discussion by Compton et al., 1992 about the NuMA protein; and Tousson et al., 1991 about a protein called centrophilin), but complementation of inactive centrosomes in *Xenopus* egg extracts occurs in the absence of microtubules, suggesting that simple interaction by diffusion is sufficient in this case. The complementation is rather slow in these extracts, however, and we have not examined whether it is faster in the presence of microtubules (Buendia et al., 1992). It is possible that *in vivo* the accumulation of proteins required for increased microtubule nucleation in prophase is accelerated by active transport, although this is not essential for the final interaction with pericentriolar material.

Several proteins that are associated with or whose concentration increases at the centrosome in prophase are found in the nucleus in interphase (Compton et al., 1992; Kuriyama, 1992; Tousson et al., 1991). This raises the possibility that *in vivo* cyclin-dependent kinases may regulate the change in microtubule-nucleating activity of centrosomes through control of nucleocytoplasmic transport of these molecules. This is not exclusive from a regulation at the level of the centrosome which must exist since the experiments we did were carried out in egg extracts free of nuclei. But this may provide an additional control that could tie the increase in microtubule-nucleating activity of centrosomes with the completion of DNA replication.

IV. BIOCHEMICAL ASSAYS FOR THE IDENTIFICATION OF PROTEINS INVOLVED IN THE REGULATION OF MICROTUBULE NUCLEATION: WHERE DO WE STAND?

With the discovery that γ-tubulin is present in most MTOCs and essential for nucleation (Horio et al., 1991; Joshi et al., 1992; Oakley, 1992; Stearns et al., 1991), it appears unnecessary to search for other molecules associated with regulation of microtubule nucleation. However, we still do not understand how the microtubule-nucleating material is organized, how it interacts with the centrioles, and how its activity is regulated during the cell cycle and cell differentiation. Moreover, biochemical methods have found other molecules to act as microtubule nucleators (Toriyama et al., 1988), and these should not be dismissed until we understand how the whole structure is organized. Specific MAPs may also play a role in nucleation. The observation that a MAP1-B-like protein is enriched in centrosomes is particularly intriguing because this protein is phosphorylated (Diaz and Avila, 1989). Therefore, a MAP1-B-like protein, like the 225 Kd protein recognized by the CHO3 antibody (Kuriyama and Maekawa, 1992), and other MAPs found at the centrosome (Kellogg et al., 1989) are candidates for the regulation of microtubule nucleation during mitosis. It is difficult, however, to determine whether a protein other than γ-tubulin is directly involved in nucleating microtubules off centrosomes without the help of genetics. It has been argued that the inhibition of microtubule nucleation off centrosomes by specific antibodies establishes the role of a protein in microtubule nucleation. We are not convinced by this argument because antibodies are bulky molecules that might inhibit microtubule nucleation through binding to proteins localized close to another protein that is essential for

nucleation. Apart from the genetic approach that was based on the visual screening of mutants showing defects in microtubule organization, biochemical approaches have been used in yeast, mammalian cells, and early embryos. In yeast and animal cells, the strategy was to isolate spindle pole bodies or centrosomes and to either extract potential microtubule nucleating material by salt or produce monoclonal antibodies (Bornens, 1992; Kuriyama, 1992; Rout and Kilmartin, 1991). In early embryos, various biochemical approaches are based on the knowledge that a maternal store of MTOC and centriolar proteins exist in the eggs (Gard et al., 1990; Holy and Schatten, 1991; Kallenbach, 1985; Klotz et al., 1990; Schatten et al., 1992; Sluder et al., 1990; Weisenberg and Rosenfeld, 1975). We will only review approaches involving fractionation of the nucleating material using functional assays.

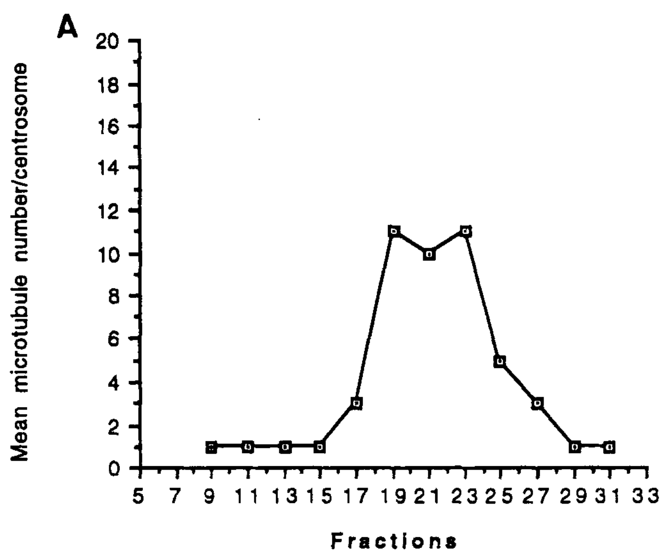
Toriyama et al. (1988) attempted to purify MTOCs from mitotic apparatus isolated from sea urchin eggs. The proteins were extracted with 0.5M KCL and found to aggregate after dialysis against low ionic strength buffer. Aggregation led to the formation of granular structures (30–100 nm) similar in appearance to the material present around the centrioles at the poles of the mitotic spindles *in vivo*. Moreover, these aggregates had the capacity to nucleate microtubule asters. One major protein of 51 kDa was found in the aggregate. Specific antibodies raised against this protein showed that it was stored in the cytoplasm of the unfertilized sea urchin egg, absent in the spermatozoa, but concentrated at the center of the sperm aster following fertilization (Ohta et al., 1988). Interestingly the 51-kDa protein was also detected at the center of cytasters of microtubules induced by hexyleneglycol in sea urchin eggs (Endo et al., 1990). The 51-kDa protein has homologies with EF1α and binds GTP (Ohta et al.,1990). Sakai and Ohta (Sakai and Ohta, 1991) believe that the 51-kDa protein has a role in the initiation of astral and spindle microtubules and that its activity is regulated by a GDP/GTP exchange factor, which is itself regulated by phosphorylation. The equilibrium shifts toward GTP-liganded 51-kDa protein during mitosis would lead to increased microtubule nucleation. It is not yet clear whether this protein is present only in sea urchin eggs or if homologous proteins exist in other species.

In yeasts, the characterization of the MTOCs (SPB) began with the preparation of fractions largely enriched (600 times that of whole yeast cell proteins) in SPBs active at nucleating microtubules *in vitro* (Rout and Kilmartin, 1990). Yamamoto et al. (1990) observed that some proteins could be solubilized from the SPB by incubation in 1M KCL

that aggregated upon dialysis against a low ionic strength buffer. These aggregates were able to nucleate microtubules in pure tubulin. Although they did not identify the proteins involved in this process, this result is reminiscent of that obtained with sea urchin microtubule nucleating material. Unfortunately, it has been impossible to do such experiments with purified mammalian centrosomes (Bornens et al., 1987; Klotz et al., 1990).

An alternative approach is to use the complementation assay described in Figure 3 with fractions purified from egg extracts or immunodepleted of a specific protein from the extract. This is an interesting approach because *Xenopus* eggs do not contain centriolar structures and it is the sperm centriole that serves as an elementary structure to initiate the formation of the zygote centrosome from microtubule-nucleating material stored in the egg. In initial experiments, we found that the material that complements inactive centrosomes (Figure 3) remains in solution after centrifugation at 250,000 ×g for 1 hour (Buendia et al., 1992). In

Figure 4. Fractionation of the microtubule nucleating activity by functional complementation. A 250,000 ×g supernatant obtained from crushed interphasic *Xenopus* eggs was subjected to ammonium sulfate fractionation (30%). The ammonium sulfate pellet obtained from 2.5 ml of crude extract was resuspended in 200 µl of acetate buffer (100 mM CH_3COOK, 2.5 mM CH_3COOMg, 30 mM EGTA) and dialysed against the same buffer. The soluble proteins were then loaded on a Superose 6 column and 500 µl fractions were collected. Fractions were pooled by batches of 2 and tested for their capacity to complement the nucleating activity of urea-inactivated centrosomes (see Figure 3). The nucleating activity of the centrosomes was assayed in a solution of pure tubulin purified on phosphocellulose (Bré and Karsenti, 1990) and adjusted to 20 µM. (A) mean microtubule number nucleated per centrosome after complementation by the different fractions. (B) analysis of the fractions by SDS PAGE (5%). The gel was silver stained, molecular weight markers are in kDa. The column fractions containing activity were between the arrowheads. The apparent molecular weights indicated have been calculated from a standard curve obtained with thyroglobulin (669,000); ferritin (440,000); β-amilase (200,000); Bovine serum albumine (67,000) and carbonic anhydrase (29,000) passed over the same column.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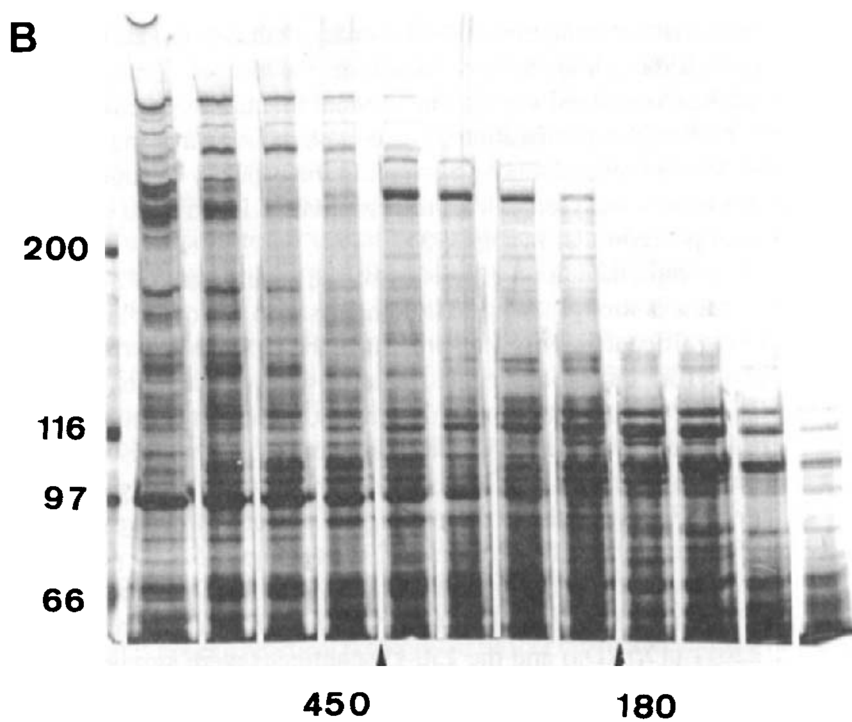
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order to further purify the component involved in the complementation of the centrosomes, the 250,000Xg supernatant was fractionated by ammonium sulfate. A large fraction of the activity was found to precipitate in 30% ammonium sulfate. Some activity could be recovered after resuspension and dialysis. This fraction was then applied to a Superose 6 column to determine the approximate size of the active material and purify it further. The different fractions (grouped by batches of two) were then tested for their capacity to complement inactivated centrosomes. Six positive fractions were found with an apparent molecular weight of 240 kDa (Figure 4). The centrosomes complemented with the crude 250,000Xg supernatant did nucleate about 26 microtubules per centrosome on average in a solution of pure tubulin at 10 μM. The centrosomes complemented with the Superose fractions nucleated only 11 microtubules in a solution of tubulin at 20 μM (Figure 4A), suggesting that the activity was very dilute in the column fractions. Analysis of the protein composition of these fractions on polyacrylamide gels is shown in Figure 4B. The protein pattern is quite specific but still highly complex. Unfortunately centrosomal proteins had a strong tendency to aggregate during dialysis, although apparently soluble in the extract. This aggregation, which was worsened during the ammonium sulfate precipitation step, hampered further purification of the complementing material in a soluble form. We discovered this by examining the capacity of supernatant and pellets from a dialysed extract to complement inactivated centrosomes (similar procedure as in Figure 3).

The complementation efficiency of the supernatant of a centrifuged dialysate was strongly reduced in comparison to that of the initial extract, and a significant activity was present in the pellet. Moreover, when we incubated directly the aggregated material with pure tubulin (10–15 μM), adding no centriolar structures in the assay, numerous microtubule asters were formed. We verified by electron microscopy that this material did not contain centrioles, and in fact, it looked very much like pericentriolar material. Finally, at least three antigens present in centrosomes were found at the center of these asters: CTR2611 (Figure 5A,B) γ-tubulin (not shown) and a 250-kDa protein recognized by a monoclonal antibody raised against high molecular weight rat brain MAPs (Figure 5C,D). The CTR2611 (170 kDa) and the 250-kDa antigens were strongly enriched in concentrated fractions of aggregated material as shown in the immunoblot of Figure 6. Unfortunately, these fractions of aggregated material contained many other proteins that were impossible to delete, suggesting that nonspecific precipitation of several proteins had occurred

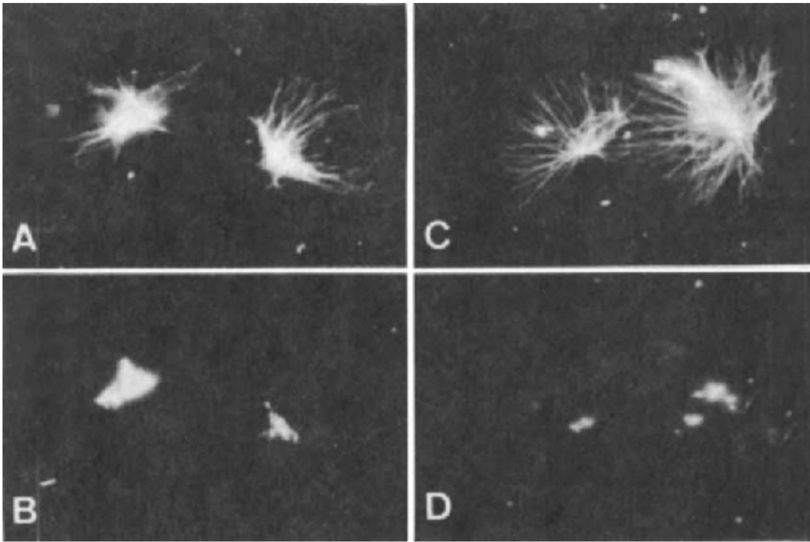

Figure 5. The centrosomal antigen CTR2611 and a 250-kDa protein recognized by a monoclonal antibody raised against high molecular weight rat brain MAPs (IBO antibody) aggregate with microtubule-nucleating material in dialyzed egg extracts. A 250,000 ×g egg extract supernatant was dialyzed against acetate buffer for 30 min at 4°C and centrifuged on a sucrose gradient for 20 min. at 100,000 ×g. A particulate material was recovered at the 40%–70% sucrose interface that was not present when an undialyzed extract was centrifuged under the same conditions. Upon incubation of the material present at the interface with pure calf brain tubulin, asters formed that are visualized here by immunofluorescence (A,C, see Buendia et al., 1992 for technical informations on immunofluorescence). The center of these asters was specifically stained by the CTR2611 monoclonal antibody (B) and IBO antibody (D).

or that complex aggregates of pericentriolar material form in dialysed *Xenopus* egg extracts in contrast to what happens in sea urchin egg extracts. In any case, this poses a practical problem for further biochemical fractionation that probably can be overcome but still requires some work.

The finding that the activity complementing inactive centrosomes in egg extracts is soluble in its native state is of interest. This suggests that in *Xenopus* the maternal store of nucleating material is in a soluble form

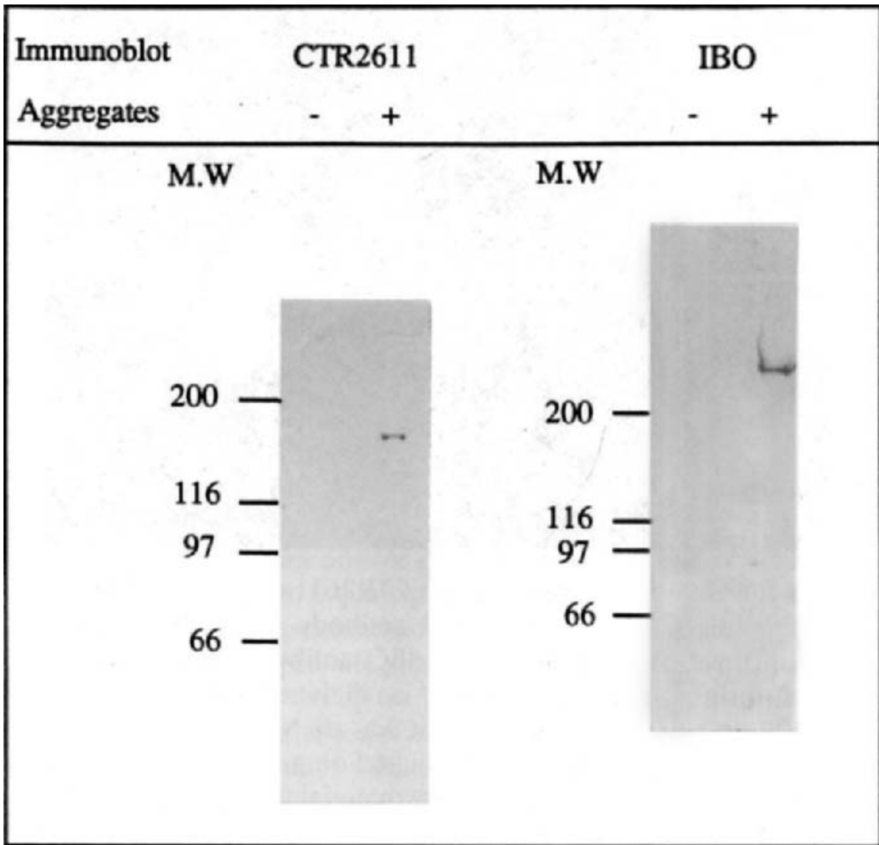

Figure 6. Detection of a 170-kDa protein by the CTR2611 antibody and a 250-kDa protein by IBO antibody in the aggregated microtubule-nucleating material. The 40%–70% sucrose interface of gradients loaded with dialysed (+) and undialysed (–) extracts were collected, loaded on polyacrylamide gels and, after transfer to nitrocellulose, proteins were detected by immunoblotting with the CTR2611 antibody and the IBO antibody.

and becomes tightly bound to the sperm centrosome upon fertilization. A tendency to aggregate seems to be a general feature of microtubule-nucleating factors. We found in egg extracts the aggregation that occurs upon dialysis could be inhibited by addition of very high concentrations of EGTA (20–60mM) to the dialysis buffer or by micromolar concentrations of GTPγs (unpublished results). This suggests that Ca^{2+} ions and G proteins may be involved in the regulation of the state of the nucleating

material and may be in its interaction with centrioles. Presently however, these observations represent nothing more than circumstantial evidence that need to be addressed in more detail after the identification of relevant molecules.

We now have two molecules, the 170-kDa antigen recognized by the CTR261 antibody (Buendia et al., 1990) and the 250-kDa protein recognized by the anti-MAP1B antibody, which are found in the pericentriolar material *in vivo* (Dominguez et al., 1994; Andersen et al., 1994) and coaggregate with microtubule-nucleating material. We will focus on the characterization of these molecules. The centrosome is obviously an extremely complex structure. Interesting questions concerning the regulation of microtubule nucleation and the interaction of specific proteins of the MTOC with the centriole cylinders await an answer. There are changes in the state of these molecules when they transit from a soluble state in the cytoplasm to an aggregated state either spontaneously or upon interaction with centrioles. It will be of interest to understand how they remain soluble in eggs, how the cyclin-dependent kinase affects their interaction with centrioles, and their capacity to nucleate microtubules, and most of all, which proteins among those that are being identified are intermediate players in the regulation of microtubule nucleation by cyclin-dependent kinases.

V. WHY IS THE NUCLEATING ACTIVITY OF CENTROSOMES INCREASED IN PROPHASE AND MITOSIS?

This is an obvious question, and yet, it is rarely addressed. Before discussing this point, it is necessary to define the concept of microtubule-nucleating activity. We envision three parameters that define the nucleating activity of a centrosome: (1) the critical tubulin concentration [c], at which they begin to nucleate microtubules, (2) the frequency of nucleation ν, and (3) the number n of microtubules that a centrosome can nucleate; at a given tubulin concentration or at a given tubulin concentration in the presence of MAPs. We have not measured ν, but we have very rough estimates of [c] and n for centrosomes incubated in an interphase and a cyclin-A-treated *Xenopus* egg extract. It turns out that [c] is barely affected by the cyclin-A-dependent kinase whereas n is increased, especially when centrosomes are assayed in pure tubulin solutions at high concentration. We have previously shown that the number of microtubules a centrosome can nucleate depends on the

concentration of tubulin and on the presence of MAPs like tau or MAP2 (Bré and Karsenti, 1990). Other MAPs affect microtubule growth in egg extracts, and in fact the conditions are such that centrosomes probably function at saturation of microtubules. It therefore makes sense that in prophase there is an increase in n rather than a decrease in [c]. Why is that? It was first thought that the increase in microtubule-nucleating activity of centrosomes would lead to an increase in polymer mass, leading to more unstable microtubules through a decrease in free tubulin dimers, thereby explaining the increased dynamics of microtubules in mitosis (Kirschner and Mitchison, 1986).

However, this cannot be the case for two reasons. First, global tubulin concentration is regulated in such a way that when polymer increases more tubulin is synthesized to cope with this change (Cleveland, 1983). Second, even if the tubulin monomer was depleted by increased polymerization at the centrosome, this would not explain the characteristics of microtubule dynamics during mitosis. Indeed, this would be expected to reduce the elongation rate of microtubules in the same time as increasing the frequency of catastrophes. and this phenomenon is not observed (Walker et al., 1988). In fact, we now know that cyclin-dependent kinases independently govern microtubule dynamics and the nucleating activity of centrosomes. (Buendia et al., 1992; Verde et al., 1992). The second idea of increasing the nucleating activity of centrosomes would increase the chance of an encounter between centrosomal microtubules and kinetochores (Kirschner and Mitchison, 1986). This is probably the best bet. In fact, increased nucleation occurs already in prophase. This is a very important period in mitosis because this is when the two centrosomes migrate around the nucleus, and it is important that centrosomes function at their maximum during this time for two reasons: (1) to allow proper antiparallel interactions between the microtubules emanating from the two centrosomes, and (2) to allow interactions with cortical cues that govern orientation of spindle axis in response to cortical signals (Hyman and Stearns, 1992; Verde et al., 1992).

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THE ESSENTIAL ROLES OF CALCIUM DURING MITOSIS

Robert M. Tombes and Gary G. Borisy

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ABSTRACT

Calcium plays significant and distinct roles in three stages of mitosis: the onset of mitosis (nuclear envelope breakdown), chromosome segregation (anaphase), and cytokinesis. These conclusions come from a wide variety of approaches, including direct measurements of calcium, blocking and recovery experiments using calcium chelators, pharmacological agents and antibodies, and molecular genetics. It is clear that (a) mitosis onset is dependent on calcium, (b) anaphase and cytokinesis are at least regulated by calcium, and (c) much remains to be determined about how calcium binding proteins interact with other M-phase regulators to control M-phase events leading to cell division.

I. INTRODUCTION

Transient or prolonged elevations in Ca^{2+} stimulate diverse cellular phenomena, including muscle contraction, neurotransmitter release, and metabolic activation via Ca^{2+} binding proteins (reviewed in Tsien and Tsien, 1990; Kretsinger, 1979). Within the last dozen years, from studies conducted in a wide variety of species, the list of calcium-stimulated events has grown to include certain M (mitotic)-phase events, such as the breakdown of the nuclear envelope (NEBD), the onset of chromatid separation (anaphase), and cytokinesis (reviewed in Ratan and Shelanski, 1986; Means and Rasmussen, 1988; Hepler, 1989; Whitaker and Patel, 1990; Anraku et al., 1991; Hepler, 1994). Much of our past understanding of the role of Ca^{2+} during M phase is based on inferences from the cellular locations of calcium-binding proteins such as calmodulin (CaM) and from the effects of calmodulin antagonists. Advances in video microscopy and in Ca^{2+}-specific fluorescent and luminescent dyes to directly measure and locate Ca^{2+} release (Tsien, 1989) have presented opportunities to implicate Ca^{2+} release in the regulation of specific M-phase events. Recently, definitive roles for calcium-regulating or -regulated proteins during M phase have been established using molecular genetics approaches in unicellular organisms (Anraku et al., 1991; Morris and Enos, 1992). Nonetheless, much remains to be determined about how Ca^{2+} controls both mitotic and meiotic M phases and how Ca^{2+}-dependent pathways interact with Ca^{2+}-independent M-phase pathways. This review will summarize how Ca^{2+} and its targets are believed to stimulate M-phase onset, chromatid separation, and cytokinesis and how Ca^{2+} might integrate with other M-phase regulators in rational models.

II. EVIDENCE FOR A ROLE OF CALCIUM DURING MITOSIS

A. Approaches

Both molecular genetics and video microscopy have contributed greatly towards recent advances regarding the regulation of M-phase events by Ca^{2+}. Although molecular genetics helps define the targets of Ca^{2+} and thus provides definitive indicators of the role of calcium in populations of cells, approaches that measure Ca^{2+} directly in single cells provide unique information about the site and kinetics of the Ca^{2+}-release pathway. Even so, results obtained using fluorometric or luminescent Ca^{2+} indicators must be interpreted carefully. For both technical and biological reasons, transient elevations of Ca^{2+} can be temporally, but not functionally, coupled to events of interest (see Tsien and Tsien, 1990; Tsien, 1989 and Hepler, 1989 for reviews on technical limitations). The limitations of detection of Ca^{2+}-sensing dyes may in some cases be due more to the natural superposition of Ca^{2+} source and target rather than to inadequacies in experimental technique (Silver, 1989; Tsien and Tsien, 1990; Tombes et al., 1992). In other words, Ca^{2+} flux can occur without a net increase in the cytoplasmic level of Ca^{2+} due to the proximity and tight feedback coupling between Ca^{2+}source and target. Most M-phase-associated Ca^{2+} changes are more subtle in amplitude than those associated with neuronal activity, muscle contraction, or even fertilization and are not, in general, the result of cell-surface signaling events. Nonetheless, inositol triphosphate (IP_3) has been used to precociously stimulate Ca^{2+} release during M phase (Twigg et al., 1988; Kline and Kline, 1994) and natural oscillations of IP_3 have been detected that coincide with transient elevations of Ca^{2+} (Ciapa et al., 1994). Mitotic events are typically stimulated as a result of some intracellular signal, such as the completion of DNA synthesis; Ca^{2+} release can therefore be spatially limited to specific mitotic sites, such as the nucleus or the cleavage furrow. Elevations of Ca^{2+} during M phase are both uniform throughout the cell (Poenie et al., 1986; Tombes et al., 1992) and spatially limited (Ratan et al., 1986; Fluck et al., 1991).

B. M-Phase Onset (Prophase)

M-phase onset, when the cell transforms itself from an interphase to an M-phase morphology, is one of the pivotal commitment steps or

checkpoints during the cell cycle (Hartwell and Weinert, 1989). MPF (M-phase promoting factor) is considered to be universally necessary to induce this transition in both meiotic and mitotic cells (Nurse, 1990; Murray and Kirschner, 1989; King et al., 1994). Biochemical and genetic evidence has shown that MPF acts by phosphorylating relevant mitotic substrates; MPF consists of a 34,000 Mr serine/threonine protein kinase, known as the cdc2 kinase ($p34^{cdc2}$), and a regulatory subunit, known as cyclin B. Targets of phosphorylation include chromatin-binding proteins, such as histone H1, and proteins of the nuclear lamina, such as lamin A and lamin C (Moreno and Nurse, 1990). Ca^{2+} flux and Ca^{2+}-dependent proteins have also been definitively linked to the onset of M phase.

A single transient elevation in Ca^{2+} from approximately 100 nM to as high as 500 nM and lasting approximately 1 min has been linked to nuclear envelope breakdown (within 5 min) in both mammalian and invertebrate cells, using indicator dyes (Steinhardt and Alderton, 1988; Kao et al., 1990; Tombes et al., 1992). Both NEBD and transient Ca^{2+} elevations can be blocked by excess Ca^{2+} chelators in echinoderm embryos (Schuetz, 1975; Steinhardt and Alderton, 1988; Twigg et al., 1988; Silver, 1989), mammalian embryos (Tombes et al., 1992), and mammalian cells in culture (Kao et al., 1990). Precocious NEBD can be induced in sea urchin eggs by microinjecting calcium-EGTA buffers set to free Ca^{2+} concentrations of at least 0.5 μM (5×10^{-7} M), and similar injections can rescue cells previously blocked by calcium chelators (Steinhardt and Alderton, 1988; Twigg et al., 1988). This sensitivity to calcium is possible only within a narrow temporal window preceding NEBD in sand dollar blastomeres (Silver, 1989) and sea urchin eggs (Twigg et al., 1988). This may reflect the dependence of M-phase onset on the synthesis of new protein in sea urchin embryos as a potential means to guard against precocious NEBD induced by the fertilization wave of Ca^{2+} (Twigg et al., 1988). Chromatin condensation in sea urchin embryos is also reversibly blocked by calcium chelation and can be prematurely induced by the introduction of calcium (Twigg et al., 1988).

Interestingly, meiotic M phase has consistently been shown to occur in the absence of microscopic or electrically measured fluctuations in Ca^{2+} and is not inhibited by calcium chelators in mouse, frog, and echinoderm oocytes (Robinson, 1985; Cork et al., 1987; Witchel and Steinhardt, 1990; Picard et al., 1990; Tombes et al., 1992). If calcium-dependent events occur prior to the induction of meiotic M-phase, they would have to precede meiotic nuclear envelope disassembly by a significant time, since calcium chelators are introduced 1 or more hours

before germinal vesicle breakdown (GVBD) (Witchel and Steinhardt, 1990; Tombes et al., 1992). Alternatively, there may be differences between the controls that regulate GVBD and NEBD, even though the fundamental properties and dynamics of nuclear envelopes are believed to be conserved between meiosis and mitosis (Stick, 1987). Additional signaling pathways that transduce extracellular cues to the nucleus operate in oocytes to induce oocyte maturation and GVBD (Meijer and Zarutskie, 1987); it is therefore surprising to observe potentially fewer regulatory steps at meiosis than at mitosis.

Calmodulin is a logical target of action of Ca^{2+} at the onset of mitotic M phase (Rasmussen and Means, 1989). Although membrane permeable calmodulin antagonists such as chlorpromazine and trifluoperazine can have nonspecific side effects, they are capable of blocking mitotic M phase at low concentrations (Boder et al., 1983; Eilam and Chernichovsky, 1988). At the onset of meiotic M phase, on the other hand, neither additional calcium nor calmodulin can induce GVBD (Cicirelli and Smith, 1987). These results are consistent with a model in which Ca^{2+} stimulates mitotic M phase via calmodulin while meiosis is induced in a Ca^{2+}-independent manner. A role for calmodulin in cell cycle control, particularly at the G_2–M transition, has also been found in yeast, mold, and plant cells (Anraku et al., 1991; Lu et al., 1992; Roberts and Harmon, 1992). Not only is extracellular calcium essential for M phase, but expression of the unique and essential calmodulin gene in yeast (Ohya and Anraku, 1989; Davis, 1992) and mold (Lu et al., 1992) is required for M phase.

The multifunctional calmodulin-dependent protein kinase II (CaMK), a protein kinase well studied in the brain (Hanson and Schulman, 1992), has been found to be the essential downstream target of Ca^{2+} and calmodulin at mitotic M phase (Baitinger et al., 1990). By using inhibitory oligopeptides from the autoinhibitory domain of CaMK and antibodies against the β subunit of CaMK, sea urchin eggs were blocked from undergoing NEBD but not subsequent mitotic events (Baitinger et al., 1990). Two isoforms of CaMK associate with each other in echinoderm eggs and can be activated in the same Ca^{2+}/calmodulin-dependent manner as the mammalian brain enzyme (Baitinger et al., 1990).

Unlike $p34^{cdc2}$, natural targets of CaMK, other than itself, have not been identified at M-phase onset. A 62-kDa protein associated with mitotic sea urchin spindles is phosphorylated in a Ca^{2+}/calmodulin-dependent manner and is essential for microtubule depolymerization, but

CaMK has not been definitively identified as the responsible kinase (Dinsmore and Sloboda, 1988; Dinsmore and Sloboda, 1989; Johnston and Sloboda, 1993). CaMK has been found in mold (Kornstein et al., 1992), in budding yeast (Pausch et al., 1991), and in cultured mammalian cells (Yamakawa et al., 1992) providing further information that this kinase may be found in many nonneuronal cell types to regulate M-phase onset. In mammalian cells, CaMK may regulate the cell cycle in G1, rather than at the G2/M transistion (Tombes et al., 1995). CaMK is an ideal target for the transient Ca^{2+} elevations frequently seen at the onset of M phase, because a brief interaction with Ca^{2+}/calmodulin induces longer-lasting activation by autophosphorylation (Schulman et al., 1992; Hanson and Schulman, 1992).

In addition to substrates of Ca^{2+}-regulated kinases, there are other proteins that might mediate the effect of Ca^{2+} at M-phase onset. CDC31 is a yeast (*Saccharomyces cerevisiae*) protein that has Ca^{2+}-binding domains and is responsible for spindle pole body duplication (Baum et al., 1986). Although calmodulin has also been reported at the centrosome and along spindle fibers (Andersen et al., 1978; Keith, 1987; Zavortnik et al., 1983; Sweet et al., 1989) and calmodulin deficient yeast show abnormal chromatin (Davis, 1992), neither the nuclear lamina nor chromatin has been shown to contain targets of Ca^{2+}-dependent phosphorylation or Ca^{2+}-binding proteins.

There are a few reports that have linked MPF and Ca^{2+} at M phase. When PSTAIR, a peptide representing a conserved $p34^{cdc2}$ kinase domain, is injected into GV-stage starfish oocytes, internal Ca^{2+} release is stimulated (Picard et al., 1990). Although Ca^{2+} release induced by the PSTAIR peptide (Picard et al., 1990) or by other means (Tombes et al., 1992; Witchel and Steinhardt, 1990; Cork et al., 1987) does not induce GVBD, this effect of a noncatalytic domain of $p34^{cdc2}$ may represent a potential *feedforward* mechanism from MPF to Ca^{2+}. Ca^{2+}, acting through CaMK, also inactivates MPF in both sea urchin (Suprynowicz et al., 1994) and *Xenopus* (Lorca et al., 1993) eggs. CaMK has also been reported to cycle in activity and associate with $p34^{cdc2}$ in sea urchin embryos (Tombes and Peppers, 1995).

One interpretation of results that implicate both Ca^{2+} and MPF in M-phase induction is that calcium may represent a *parallel, essential pathway* to $p34^{cdc2}$ (MPF). Evidence for a parallel M-phase inducing pathway to $p34^{cdc2}$ comes from the filamentous fungus, *Aspergillus nidulans* (Osmani et al., 1991), where the activity of another serine/threonine protein kinase, $p79^{nimA}$, is equally essential to $p34^{cdc2}$ to

initiate mitosis (Osmani et al., 1991). Feedforward signaling between these two apparently essential kinases may be implied by the result that premature mitosis is induced when either $p79^{nimA}$ or $p34^{cdc2}$ is hyperactivated or overexpressed (Russell and Nurse, 1987; Osmani et al., 1988).

C. M-Phase Progression (Prometaphase and Metaphase)

Once a cell has reached prometaphase, that is, the nuclear envelope has broken down and chromosomes are condensed but have not yet aligned on the metaphase plate, there does not appear to be a strict Ca^{2+} requirement to achieve the metaphase configuration. CaMK is unimportant at events beyond nuclear envelope breakdown (Baitinger et al., 1990). Prometaphase cells injected with Ca^{2+} chelators are not prevented from forming a mitotic spindle and aligning chromosomes on the metaphase plate (Tombes and Borisy, 1989; Kao et al. 1990); however, cells do not proceed beyond metaphase under such low Ca^{2+}-clamped conditions. It is not known whether microtubule dynamics in prometaphase or metaphase cells are altered under these conditions of low intracellular Ca^{2+}.

Microtubules are known to be profoundly influenced by Ca^{2+} both *in vitro* (Weisenberg, 1972) and *in vivo* (Kiehart, 1981). Microtubule depolymerization can also be induced by excess calmodulin, which has been found in association with the spindle (Andersen et al., 1978; Keith, 1987; Zavortnik et al., 1983; Sweet et al., 1989). Calmodulin antagonists slow M phase (Boder et al., 1983). It is assumed that calmodulin acts on microtubules to mediate aspects of microtubule dynamics, which are important for proper chromatid capture and alignment. The 62-kDa Ca^{2+}/calmodulin protein kinase substrate is the only candidate calmodulin target known to mediate aspects of spindle microtubule dynamics (Dinsmore and Sloboda, 1988; 1989), although the mechanistic details are vague.

At metaphase it is possible that microtubule dynamics are slowed when intracellular Ca^{2+} is low such that the efficiency of chromatid capture is decreased, even though most chromatids are captured and the spindle *appears* normal. This would serve as an alternative explanation for delays of anaphase onset caused by low intracellular Ca^{2+} (see next section), since anaphase will not commence until all chromosomes have been captured (Nicklas and Kubai, 1985; McIntosh and Koonce, 1989).

D. Chromosome Segregation or Karyokinesis (Anaphase)

A Ca^{2+} *trigger* to induce anaphase onset has been a popular model because anaphase represents a major irreversible commitment step during the cell cycle. In a wide variety of cells, Ca^{2+} injected at µM levels has been reported to precociously stimulate anaphase onset (Izant, 1983; Silver, 1989; Zhang et al., 1990, Schollmeyer, 1988), while excess Ca^{2+} chelators can reversibly block anaphase onset (Izant, 1983; Hepler, 1985; Wolniak and Bart, 1985a; Silver, 1989; Zhang et al., 1990; Tombes and Borisy, 1989). The advent of specific Ca^{2+}-sensing dyes allowed studies to be conducted where sudden releases of Ca^{2+} could be temporally correlated with chromatid separation. Tight correlations of transient elevations of Ca^{2+} (transients) with anaphase onset were reported in the mammalian cell line, PtK1 (Poenie et al.,1986), and in polyspermic sea urchin embryos (Poenie et al., 1985). Significant Ca^{2+} transients were frequently seen in association with anaphase onset in PtK2 cells, but it was concluded from a population-based study that their timing made it unlikely that they triggered anaphase (Ratan et al., 1988). Swiss 3T3 cells exhibited dramatic, repetitive Ca^{2+} transients not only during M phase but also during interphase (Tombes and Borisy, 1989; Kao et al., 1990). More gradual and longer-lasting Ca^{2+} elevations that eventually returned to baseline have been associated with, but do not necessarily precede, anaphase onset in PtK2 cell lines (Ratan et al., 1986), in endosperm cells from the African blood lily, *Haemanthus* (Keith et al., 1985), in stamen hair cells of *Tradescantia* (Hepler and Callaham, 1987), and in Swiss 3T3 cells (Tombes and Borisy, 1989; Kao et al., 1990).

The role of a visible Ca^{2+} transient as the trigger for anaphase has been questioned because Ca^{2+} transients can be eliminated by removal of extracellular serum or calcium without preventing or slowing normal mitotic kinetics (Tombes and Borisy, 1989; Kao et al., 1990). Nonetheless, Ca^{2+} chelators prevent the onset of anaphase and the gradual elevation of Ca^{2+} (Tombes and Borisy, 1989; Hepler and Callaham, 1987). In *Tradescantia* stamen hair cells, where mitotic calcium is provided from external sources, Ca^{2+} channel agonists can stimulate anaphase onset while antagonists block anaphase onset (Hepler, 1985; Wolniak and Bart, 1985a; 1985b; Chen and Wolniak, 1987). Ca^{2+} flux—not detectable Ca^{2+} transients—therefore appears to be necessary for anaphase onset. Ca^{2+} transients in mammalian cells may be more closely associated with G_1 cell cycle decision events where Ca^{2+} flux is again required (Means and Rasmussen, 1988; Berridge, 1995). As suggested

before, this gradual elevation of Ca^{2+} may reflect an increased Ca^{2+} flux surrounding the metaphase–anaphase transition, which is necessary to stimulate microtubule dynamics in order to increase the efficiency of chromatid capture. At anaphase onset, therefore, Ca^{2+} is not acting as a direct trigger but is nonetheless important to ensure normal karyokinesis.

In support of the trigger model, however, calpain II represents an identified anaphase target of Ca^{2+}. Calpain II is a Ca^{2+}-dependent protease that is triggered directly by high levels of Ca^{2+}. When calpain II is injected into metaphase cells, it precociously and specifically induces anaphase onset (Schollmeyer, 1988). One of its targets is the *c-mos* protooncogene product, a protein kinase whose destruction alleviates meiotic arrest at metaphase (Sagata et al., 1989; Watanabe et al., 1989; Hunt, 1989). How *c-mos* and calpain function during M phase in somatic cells remains undetermined.

E. Cell Cleavage (Cytokinesis)

There have been more assumptions than actual proof that cytokinesis, being an actinomyosin-based event, is Ca^{2+}-dependent. Models have been proposed that by analogy to muscle implicate the calmodulin-activated myosin light chain kinase as the Ca^{2+} target in dividing cells (Mabuchi, 1986). It is a reasonable extrapolation since myosin ATPase activity, when extracted from cells in culture, can be activated via its light chain kinase in a Ca^{2+} and calmodulin-dependent manner (Yerna et al., 1978; Scordilis et al., 1977). However, although calmodulin associates with the mitotic spindle and intercellular bridge (Andersen et al., 1979), it has not been found in the cleavage furrow (Welsh et al., 1978; Hamaguchi and Iwasa, 1980). Interestingly, in *Dictyostelium discoideum*, when calmodulin expression is repressed, but not completely eliminated, the midbody or intercellular bridge fails to break, but cleavage furrow formation occurs normally (Liu et al., 1992).

Nonetheless, a role for Ca^{2+} at cytokinesis has been supported by (a) blocking cytokinesis with Ca^{2+} chelators, (b) precociously stimulating cytokinesis with Ca^{2+} ionophore, and (c) measuring Ca^{2+} changes along the cleavage furrow. Ca^{2+} buffer injections block cytokinesis, not karyokinesis in *Xenopus laevis* eggs and blastomeres (Baker and Warner, 1972; Miller et al., 1993; Snow and Nuccitelli, 1993). As in most other such approaches, which have used chelators to block M- phase events, between 1 and 2 mM final EGTA was required to achieve the blockade, which is consistent with the extensive Ca^{2+} stores within the egg

(Terasaki and Sardet, 1991). The introduction of Ca^{2+} in frog eggs (Hollinger and Schuetz, 1976) and caffeine to induce Ca^{2+} release in sea snail (*Ilyanassa obsoleta*) eggs (Conrad et al., 1987) have been reported to precociously induce cleavage furrow formation and accelerate furrow elongation.

Measurements of changes in Ca^{2+} during cytokinesis include the use of recombinant aequorin (Shimomura, 1990) to detect changes in free Ca^{2+} in large eggs such as those from the fish, *medaka*. It has been estimated that during cleavage in *medaka* eggs, Ca^{2+} is released as a slow wave, is restricted to the cleavage furrow, and peaks at 5–8 μM over a 5–10 min period (Fluck et al., 1991). PtK_2 and Swiss 3T3 cells in culture, using microfluorometric measurement of Ca^{2+} with fura-2, frequently showed Ca^{2+} transients at or near cytokinesis (Ratan et al., 1988; Tombes and Borisy, 1989); however, interphase cells also showed this behavior. Rink and coworkers, however, were unable to detect changes in Ca^{2+} using Ca^{2+}-specific microelectrodes (Rink et al., 1980).

In addition to calmodulin-mediated targets of Ca^{2+}, protein kinase C has been reported as a locus of Ca^{2+} action at cytokinesis. Activators of protein kinase C have been reported to induce unusual and transient cleavage furrows in *Xenopus* eggs (Bement and Capco, 1989). Whether protein kinase C is actually involved in cytokinesis is questionable. Calmodulin may play a role in septa formation in plant cells, (Roberts and Harmon, 1992).

III. MODELS OF CALCIUM ACTION DURING MITOSIS

Certain models integrating Ca^{2+} with cellular events have incorporated the concept of "invisible Ca^{2+} transients" (Tsien and Tsien, 1990) to explain the unexpected absence of a change in Ca^{2+}. Single-cell studies of Ca^{2+} changes using either luminescent or fluorometric dyes will yield heterogeneous results. This has been particularly true in the field of mitotic Ca^{2+} and can be exasperating to interpret (Hepler, 1989). Although Ca^{2+} imaging is fraught with potentially serious artifacts that have led to misinterpretations of Ca^{2+}-release pathways (Hepler, 1989; Tsien, 1989; Tsien and Tsien, 1990), heterogeneity in the amplitude and frequency of detectable Ca^{2+} transients may reflect the biophysical properties of Ca^{2+} release, diffusion, and binding as well as the nature of the interaction of Ca^{2+}-dependent pathways with other pathways. For example, if the distance over which Ca^{2+} diffusion takes place is small, Ca^{2+} transfer from a source to its target can take place (flux) without an

increase in free cytoplasmic Ca^{2+} due to the tight coupling that exists between the occupation of the relevant Ca^{2+} target and Ca^{2+} channel gating (Meissner, 1986). In such a case, there would be no detectable global Ca^{2+} elevation, despite a net flux of Ca^{2+} from source to target. The most sensitive detectors may be capable of measuring such local changes, however. The mitotic spindle is a potential example of the juxtaposition of Ca^{2+} source and target, since extensive calciosomes intercalate the spindle and are the relevant source of Ca^{2+} for calcium-dependent mitotic events (Silver, 1986; Hafner and Petzelt, 1987). Such compartments are not impermeable to soluble molecules such as Ca^{2+} detectors or chelators, which in sufficient excess can still block Ca^{2+}-dependent events, such as those that occur during mitosis (Whitaker and Patel, 1990).

It is risky, therefore, to build a model of Ca^{2+} action at any point during mitosis based solely on the observation of transient or long-lasting elevations of Ca^{2+} in a few cells at the appropriate times. Rather, Ca^{2+}-dependent events must be established based on a combination of approaches. Criteria should include inhibition by the removal of the source of Ca^{2+} and/or the Ca^{2+} target, stimulation by the precocious delivery of Ca^{2+}, and the identification of Ca^{2+}-binding proteins that are temporally and spatially consistent with the event of interest. Models of Ca^{2+} action at mitosis have been and will continue to be strengthened by their conservation between cell types and species but must also respect the necessary differences between different M phases. What may be true for the mammalian oocyte may not necessarily be true for the stamen hair cell, although many similarities exist (Hepler, 1989).

M-phase onset (G_2-to-M or interphase-to-prophase) is universally dependent on calcium (from yeast to mammalian tissue culture cells), although much remains to be learned about how calcium-dependent pathways interact with the MPF pathway. A dual pathway model must be invoked where Ca^{2+} and MPF are both necessary but not independently sufficient to induce M phase, even though the details of how these two pathways interact, much less why there exist two pathways, remains to be determined. Meiotic M phase is the one known exception to this M-phase model since the maturation of oocytes is Ca^{2+}-independent. A further examination of differences in meiotic and mitotic calcium differences may help explain why two pathways are employed in some, but not all, M phases. A dual pathway model of M- phase onset has also been proposed from work on another mitotic protein kinase, $p79^{nimA}$ (Osmani et al., 1991). The possibility that $p79^{nimA}$ may be a target

of Ca^{2+} action acting through CaMK has support (Lu et al., 1993) and could be the best model to integrate known regulators.

A model in which dual pathways regulate M phase may explain the occurrence of Ca^{2+} transients among roughly half of the single cells in a population M-phase onset (Poenie et al., 1985; Tombes et al., 1992). In this dual-pathway model, in which both Ca^{2+} and MPF are required to trigger NEBD, the order of Ca^{2+} release and MPF activation is assumed to be random. In cases where MPF is activated before Ca^{2+} release, negative feedback control mechanisms would close Ca^{2+}-channels before enough Ca^{2+} was released to register as a transient elevation. In the other cells, where Ca^{2+} channels are activated before MPF, then Ca^{2+} would continue to be released and register as a transient elevation. This model would depend on a negative feedback control circuit from some indicator of M-phase onset to both Ca^{2+} release and MPF. This type of circuitry remains to be determined. Precise synchrony of these two essential pathways would be difficult and in fact unnecessary to achieve. Approximate synchrony would be important to allow M phase to occur within a reasonable period of time and may be achieved by interregulation by feedforward mechanisms.

Such a feedforward link between Ca^{2+} and MPF may be represented by results obtained in starfish oocytes, where a peptide modeled on the conserved PSTAIR domain of $p34^{cdc2}$ stimulates internal Ca^{2+} release without any associated effect on $p34^{cdc2}$ kinase activity (Picard et al., 1990). If full activation of MPF is related to a conformational change in $p34^{cdc2}$ to expose this PSTAIR domain, then this result could represent a feedforward mechanism from MPF to Ca^{2+}. $p79^{nimA}$ may be the critical element through which these pathways interact or through which the Ca^{2+} pathway acts.

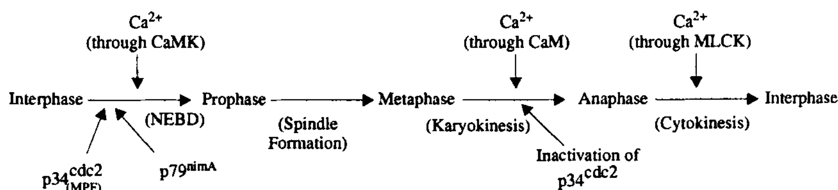
Most M phases are marked by the simultaneous induction of a diverse series of events to transform the cell from interphase into mitosis, including centrosome or spindle pole body duplication and separation, nuclear envelope disassembly, chromosome condensation, and spindle assembly. Both calcium-dependent and calcium-independent pathways have been implicated in each of these phenomena. However, in order to help explain why dual pathways of M-phase control might exist, these events are not always costimulated at M-phase onset. For example, GVBD at meiotic *M-phase onset* occurs well after chromatin has condensed into synaptonemal complexes. The transition from first meiosis to second meiosis occurs without the reformation of the nuclear envelope or the disassembly and reformation of the spindle. Clearly, the details of

the dual-pathway model of M-phase onset, including the definition of the substrates of these kinases, will shed light on how such unique M phases are achieved.

At the metaphase–anaphase transition, Ca^{2+} may not have as direct a role as it does at M-phase onset. Although Ca^{2+} removal can reversibly block anaphase onset, the nature of the effect of Ca^{2+} on microtubules implies that Ca^{2+} is necessary for spindle function but is not the ultimate stimulant of chromosome separation. The Ca^{2+} dependence of anaphase may be explained by proposing that Ca^{2+} chelation slows microtubule dynamics and thus chromatid capture, thereby preventing anaphase onset due to the presence of free chromatids. The injection of moderate levels of Ca^{2+} might precociously induce anaphase by accelerating metaphase or the capture of all free chromatids by increasing microtubule dynamics and the interaction of microtubules with kinetochores. This may explain why baseline Ca^{2+} levels slowly begin to increase around the metaphase–anaphase transition (Tombes and Borisy, 1989) and why an artificial Ca^{2+} transient does not immediately induce anaphase onset (Izant, 1983). Although the model of a dramatic, sudden Ca^{2+} release triggering the separation of chromatids at anaphase is attractive in principal, there is little supportive data. Whatever comprises the ultimate trigger of chromatid separation, it must be able to sense the association of all kinetochores with the spindle (Nicklas and Kubai, 1985) before activating topoisomerase activity (Uemura et al., 1987) or proteases (Hunt et al., 1992; Holloway et al., 1993). In agreement with this conclusion, cyclin B degradation is not dependent on Ca^{2+} (Luca and Ruderman, 1989).

Models that explain the role of Ca^{2+} at cytokinesis are based on minimal data. The assumption in mammalian cells is that myosin light chain kinase is activated via calmodulin to induce cleavage furrow formation. In plant cells, the formation of septa by the fusion of membranes may also be regulated by calmodulin. Ca^{2+} may serve more of an essential, permitting role, rather than a triggering role.

These models can be integrated into the following speculative scheme, which includes the points known to be dependent on the activation of $p34^{cdc2}$ and $p79^{nimA}$ and the inactivation of $p34^{cdc2}$. It is proposed that Ca^{2+} operates via calmodulin at all mitotic sites and perhaps through the multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaMK) at M-phase onset in embryos and myosin light chain kinase (MLCK) at cleavage furrow formation.



IV. FUTURE DIRECTIONS

In summary there are two categories within which much work remains to further clarify the role of Ca^{2+} during mitosis.

1. The identification of Ca^{2+}-binding proteins as relevant targets during various aspects of mitosis is in its infancy. At M-phase onset in some cell types, it is presumed to be calmodulin and the calmodulin-dependent multifunctional protein kinase (CaM kinase). Mitotic substrates of CaMK have not yet been identified. Calmodulin-binding proteins of the spindle and cleavage furrow are also logical targets of Ca^{2+}.
2. The activity of such Ca^{2+} targets at M-phase onset and at later mitotic stages must be integrated with the $p34^{cdc2}$/cyclin (MPF) pathway. The necessarily extensive regulation that must exist to integrate these two pathways needs to be defined. It is unknown how extensive regulation is by dual pathways or even why dual pathways might exist.

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CALCIUM AND CALMODULIN REGULATION OF THE NUCLEAR DIVISION CYCLE OF *ASPERGILLUS NIDULANS*

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Jennifer S. Dayton, and Anthony R. Means

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I. INTRODUCTION

In order to reproduce and multiply, every cell must progress through an orderly series of events, the cell cycle, consisting of mitosis and interphase. In most cells, the latter phase further includes a period of DNA replication, the S phase which is temporally separated from the previous mitosis by a *gap*, the G_1 phase, and from the subsequent mitosis by another *gap*, the G_2 phase. Elucidation of regulation of this series of events has been one of the major focuses in modern cell biology. Genetic analyses in fungi and biochemical studies in vertebrate and invertebrate species during the last decade have resulted in identification of critical regulatory proteins which specifically control progression through the decision points of the cell cycle. However, the overall process of cell proliferation is very complicated and is regulated coordinately by multiple biochemical pathways that integrate both intracellular and extracellular signals. There are many key regulatory components of these pathways that remain to be identified.

Calcium is an intracellular second messenger present ubiquitously in eukaryotic cells. It has been shown that Ca^{2+} is required for cell viability and progression through G_1/S and mitosis in mammalian cells (Whitaker and Patel, 1990; Moolenaar et al., 1985; Whitfield et al., 1980). Furthermore, it has been clearly demonstrated that many hormones, including growth factors and peptide hormones, cause transient increases in the concentration of free cytosolic Ca^{2+} by inducing either influx of extracellular Ca^{2+} into cells through voltage- or receptor-gated channels or release of Ca^{2+} from intracellular pools via the action of inositol trisphosphate (IP_3) (Rozengurt, 1986; Jacob, 1990; Berridge and Irvin, 1989). These Ca^{2+} transient signals have been shown to activate calmodulin in the cell (Hahn et al., 1992). Calmodulin is the primary intracellular Ca^{2+} receptor in eukaryotic non-muscle and smooth muscle cells (Means et al., 1991) and has been shown to be required for progression at several

specific points of the mammalian cell cycle (Means et al., 1991; Rasmussen and Means, 1987; Rasmussen and Means, 1989). However, elucidation of molecular mechanisms by which calmodulin acts has been hindered by the difficulty involved in genetic manipulation of mammalian cells where there are three calmodulin genes. These genes not only encode identical calmodulin proteins, but also are differentially regulated during the cell cycle as well as in response to extracellular signals (Bender et al., 1988; Fischer et al., 1988; Nojima, 1989; Bai and Weiss, 1991). These complications have led us and others to use lower eukaryotic organisms as model systems. Calmodulin has been shown to be essential for cell growth in three genetically tractable systems namely, *Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Aspergillus nidulans* (Davis et al., 1986; Takeda and Yamamoto, 1987; Rasmussen et al., 1990). Since there are considerable differences between mammalian and budding yeast systems in terms of Ca^{2+} and calmodulin function during the cell cycle (for review see Lu and Means, 1992a), we chose *Aspergillus nidulans* as a model. By generating strains conditional for calmodulin expression in different genetic backgrounds, we have demonstrated that both Ca^{2+} and calmodulin are rate-limiting factors in regulating the nuclear division cycle of *Aspergillus nidulans* (Lu et al., 1992) and also identified two potential molecular targets, $p34^{cdc2}$ and NIMA mitotic kinases, for Ca^{2+}/calmodulin action during G_2/M progression (Lu et al., 1993d). Our results also reveal that one essential Ca^{2+}/calmodulin dependent target is the serine/threonine protein phosphatase, calcineurin (Rasmussen et al, 1994).

In this review, we shall discuss the regulatory roles for Ca^{2+} and calmodulin in controlling the nuclear division cycle in *Aspergillus nidulans*, with an emphasis on the molecular mechanisms by which Ca^{2+} and calmodulin function during progression into mitosis. We shall also compare the roles of Ca^{2+} and calmodulin in *Aspergillus nidulans* to those in budding yeast. Comprehensive reviews on cell cycle regulation by Ca^{2+} (Whitaker and Patel, 1990) and calmodulin in mammalian cells (Lu and Means, 1992a; Means et al., 1991) or in the yeast *Saccharomyces cerevisiae* (Anraku et al., 1991) are available.

II. CALCIUM, CALMODULIN AND THE *ASPERGILLUS* NUCLEAR DIVISION CYCLE

The following features make the filamentous fungus *Aspergillus nidulans* an excellent model system for cell cycle studies. It is an eukaryotic

organism with sophisticated genetics, a well-marked genetic map and defined nutritional requirements (Cove, 1977; Timberlake and Marshall, 1989). It is normally grown as a haploid so that it is amenable to introduction and subsequent identification of mutations, but can also be grown as a diploid, making it possible to determine genetically whether different mutations are in the same gene. In *Aspergillus nidulans*, DNA-mediated, site-specific integrative transformation occurs at high fre-

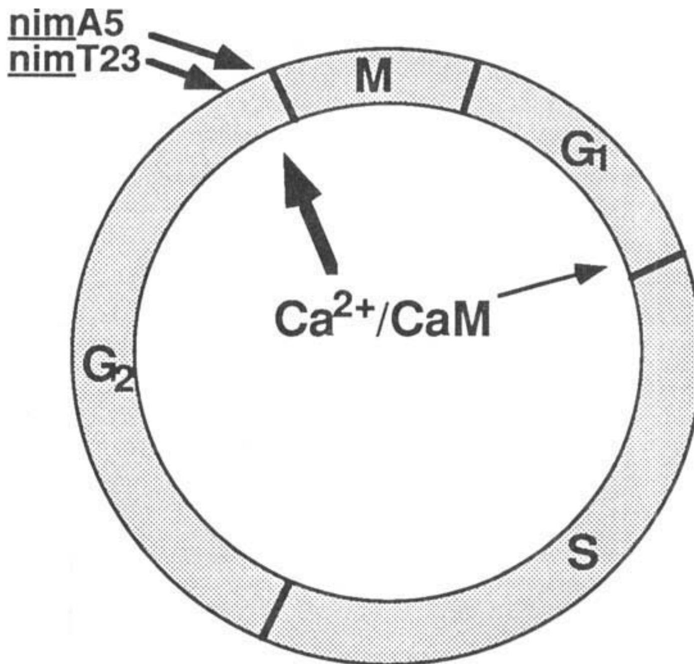


Figure 1. Illustration of the *Aspergillus nidulans* nuclear division cycle and control points sensitive to temperature-sensitive *nim*T23 and *nim*A5 as well as *alc*A-regulated calmodulin mutations.

The *Aspergillus nidulans* nuclear division cycle consists of four phases, 15 min G_1, 40 min S, 40 min G_2 and 5 min M. Progression through this cycle can be blocked at specific points by temperature-sensitive mutations, such as *nim*A5 and *nim*T23 which arrest cells in G_2 at the restrictive conditions, or by reducing intracellular calmodulin levels. In the presence of low calmodulin conditions, 85% of cells are arrested in G_2 with the remaining in G_1 and/or S.

quency and a defined inducible expression system is available (Timberlake and Marshall, 1989; Waring et al., 1989). Thus it is possible to clone genes important for cell cycle progression by complementation of conditionally lethal mutant phenotypes, then to mutate, replace, overexpress or repress the cloned genes to study their functions as well as to analyze the structure-function relationships of essential genes *in vivo* (Morris et al., 1989). Moreover, site-specific gene disruption allows one to destroy a gene and then to analyze the effect of the resulting null mutation on cell function. The organization of the nuclear division cycle in *Aspergillus nidulans* offers another attractive feature for study of eukaryotic cell cycle control. The nuclear division cycle is about 100 min and consists of four phases similar to the mammalian cell cycle, a 15 min G_1, 40 min S, 40 min G_2 and 5 min M (Figure 1) (Bergen and Morris, 1983). Many temperature sensitive mutant strains have been isolated by Morris which arrest cells at specific points of the nuclear division cycle at the restrictive temperature (Morris, 1976). Recent characterization of some of these mutations has revealed that regulatory mechanisms of the *Aspergillus nidulans* cell cycle are highly conserved when compared to those identified in mammalian and other eukaryotic cells (Morris, 1990; Osmani et al., 1991a). For all these reasons, we have chosen *Aspergillus nidulans* to address the molecular mechanisms by which Ca^{2+} and calmodulin regulate the cell cycle.

A. Creation of Strains Conditional for the Expression of Calmodulin in Different Genetic Backgrounds

In order to initiate studies on the influence of calmodulin on cell growth in *Aspergillus nidulans*, we isolated and sequenced complete cDNA and genomic clones for the unique calmodulin gene present in this organism (Rasmussen et al., 1990). The gene encodes a protein with 84% identity (93% similarity) to vertebrate calmodulin. Bacterially expressed and purified *Aspergillus nidulans* calmodulin binds 4 Ca^{2+} ions and activates three vertebrate calmodulin-dependent enzymes with kinetics similar to its vertebrate counterpart (Rasmussen et al., 1990). Substitutions of the glutamic acid residue that occupies the 12th position of each Ca^{2+}-binding loop with valine completely abolishes the ability to bind Ca^{2+} or to activate calmodulin-dependent enzymes isolated from either mammalian sources or *Aspergillus nidulans* (Lu, K. P. & Means, A. R., unpublished data). Bacterially produced calmodulin was used to produce and affinity purify antibodies specific for the *Aspergillus nidulans* pro-

tein. The antibodies were employed to develop a highly sensitive and specific radioimmunoassay in order to quantify calmodulin during the growth cycle of the fungus.

To determine if calmodulin is involved in progression through the nuclear division cycle of *Aspergillus nidulans*, we first determined whether calmodulin and calmodulin mRNA were regulated during the nuclear division cycle, as is the case in mammalian cells (Rasmussen et al., 1990; Lu et al., 1993d). When quiescent conidia (dormant spores) were stimulated to enter the cell cycle, calmodulin mRNA increased nearly 20-fold, peaking at the start of S phase and then decreased by half as cells progressed through S+G_2/M. In contrast, calmodulin levels increased 2-fold prior to the onset of S phase and a further 2-fold temporally correlated with mitosis. Whereas the first increase in calmodulin is very similar to what occurs in mammalian cells, the apparent increase accompanying mitosis was unprecedented. To examine this G_2/M increase more precisely, we utilized strains harboring the *nim*A5 or *nim*T23 temperature sensitive mutation to first arrest cells in G_2 and then, by a shift to the permissive temperature, allow them to synchronously proceed through nuclear division (Lu et al., 1993d). When the cells were released from the G_2 block, *nim*A5 cells entered into mitosis in 5–10 min while *nim*T23 cells required 20–30 min as monitored by changes in the chromosome mitotic index (CMI). These CMI changes occurred precisely in concert with changes in calmodulin levels in both strains (Lu et al., 1993d). The rapid increase and decrease in calmodulin as cells entered into and completed mitosis were not accompanied by changes in calmodulin mRNA levels. Upon completion of mitosis, a second increase in calmodulin was observed that was coincident with changes in histone H3 mRNA. This latter increase in calmodulin at the G_1/S boundary was accompanied by comparable increases in calmodulin mRNA. When the non-temperature-sensitive strain R153 was examined under the same experimental conditions, there was no change in either CMI or calmodulin levels following the temperature shift. These results indicate that progression into mitosis in *Aspergillus nidulans* is associated with a unique and rapid increase in the level of calmodulin that appears to be regulated post-transcriptionally. On the other hand, exit from mitosis is accompanied by a rapid decrease in calmodulin that is reminiscent of the catastrophic degradation of cyclin B (Evans et al., 1983; Swenson et al., 1986; Murray et al., 1989). It will be fascinating to investigate the mechanisms that underlie both of these acute changes in calmodulin concentration.

Disruption of the unique calmodulin gene is lethal and therefore can only be performed in a heterokaryon in *Aspergillus nidulans* (Rasmussen et al., 1990). Therefore, to determine whether calmodulin is a rate-limiting factor for cell cycle progression, it was important to generate strains in which the level of intracellular calmodulin could be experimentally manipulated. Three different strains that are conditional for calmodulin expression were generated in either wild type, *nim*T23 or *nim*A5 genetic backgrounds. These strains were obtained by transforming GR5, *nim*T23 and *nim*A5 strains of *Aspergillus nidulans* with a pAL-CaMKP plasmid containing a portion of the *Aspergillus nidulans* calmodulin gene lacking the 3′ end of the amino acid coding region under the control of the inducible alcohol dehydrogenase (*alc*A) gene promoter (Lu and Means, 1993d) (Figure 2). When the pAL-CaMKP was integrated into the genome by site-specific homologous recombination (Figure 2), two copies of calmodulin genes were generated. One is under the control of the endogenous calmodulin promoter but is nonfunctional due to a 3′ deletion as shown by gene disruption experiments (Rasmussen et al., 1990), and the other has full coding capacity but is under the control of the *alc*A promoter. Strains satisfying these criteria were selected and named AlcCaM, AlcCaM/T23 or AlcCaM/A5, with reference to their parent strains wild type GR5, *nim*T23 or *nim*A5, respectively.

The activity of the *alc*A promoter is regulated by the carbon source present in the culture medium (Waring et al., 1989; Lu and Means, 1993d). Threonine and/or ethanol are inducers, acetate and/or glucose are repressors, and glycerol is a derepressor (non-repressing, non-inducing) that permits a low constitutive level of expression from the gene (Lu et al., 1992). In inducing medium, calmodulin mRNA levels rapidly increased over 100 fold, while the protein increased about 4 fold, and both remained at high levels for at least 24 hours, as compared with values obtained in derepressing medium. In the presence of a repressor, there was no detectable calmodulin mRNA and calmodulin levels decreased to about 5% of the normal levels by 9 hours of incubation. When the repressing medium was washed out and replaced with inducing medium, calmodulin concentrations increased rapidly, reaching maximally induced levels in 3.5 hours. In the presence of glycerol, the calmodulin level was very similar to that found in the wild type R153 strain. There were no significant differences in the response to the alternate carbon sources in the three strains containing the AlcCaM gene. Thus the expression of calmodulin can be manipulated experimentally in these strains.

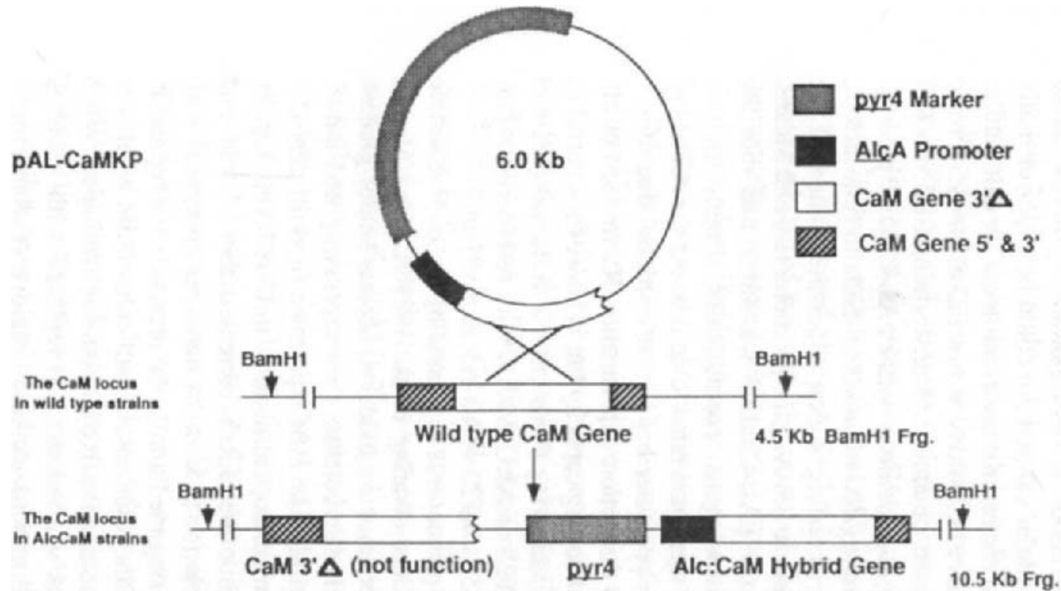


Figure 2. Site-directed homologous recombination of the pAL-CaMKP vector.

The pAL-CaMKP vector contained the *Aspergillus nidulans* calmodulin gene with a 3′ truncation under the control of to the *alc*A promoter and the selectable nutritional marker *pyr*4 gene. When this DNA was integrated into the calmodulin locus of the genome in *Aspergillus nidulans* by homologous recombination confirmed by genomic Southern analysis, two copies of calmodulin genes were generated; one contained the defective 3′ truncated calmodulin gene regulated by the endogenous calmodulin promoter and the other contained a full coding capacity but was under the control of the *alc*A promoter.

B. Cooperation between Ca^{2+} and Calmodulin in Regulating Cell Proliferation

Increased levels of calmodulin accelerate cell cycle progression in mammalian cells, but have no effect on cell growth in budding yeast. We wanted to determine which situation exists in *Aspergillus nidulans* (Lu et al., 1992). When AlcCaM cells were grown in inducing medium and calmodulin levels increased 4 to 5 fold, cells enter the nuclear division cycle earlier and dry weight increased at a greater rate than those in derepressing medium, suggesting that the rate of growth increases when calmodulin is overexpressed. Furthermore, this increase in growth rate was accompanied by an increase in the number of nuclei per cell indicating that the length of the nuclear division cycle is shortened (Lu et al., 1992). Similar results were also obtained with both AlcCaM/A5 and AlcCaM/T23 strains. These results suggest that increasing calmodulin levels allow *Aspergillus nidulans* cells to enter the cell cycle more quickly and also shortens the length of the nuclear division cycle, resulting in an overall increase in the rate as well as the extent of growth. Therefore, *Aspergillus nidulans* is more similar to mammalian cells than budding yeast relative to the effects of calmodulin concentration on cell growth rate.

Since cell proliferation depends on the calmodulin concentration in *Aspergillus nidulans*, and the *Aspergillus nidulans* calmodulin requires Ca^{2+} to function *in vitro*, we questioned if extracellular Ca^{2+} is required for cell growth (Lu et al., 1992). When cells were incubated in media containing different concentrations of Ca^{2+}, we found that *Aspergillus nidulans* requires extracellular Ca^{2+} for growth. When incubated in 2 nM Ca^{2+} (the lowest concentration of Ca^{2+} we could achieve), cells ceased growing after 1 to 2 nuclear division cycles. The concentration of Ca^{2+} required for half maximal growth is 3 to 4 μM and optimal growth occurs at 10 μM. Since cell growth does not occur in response to the addition of other metals such as Mg^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, or Zn^{2+}, this ion requirement for growth appears to be Ca^{2+} specific (Lu et al., 1992). Since yeast cells can grow indefinitely in the absence of extracellular Ca^{2+}, this may be the first demonstration that extracellular Ca^{2+} is essential for growth of lower eukaryotic cells. However, a note of caution must be introduced since our experiments used EGTA buffer systems to experimentally manipulate the extracellular Ca^{2+} concentration. Similar studies in other fungi have led to the conclusion that extracellular Ca^{2+} is not required for growth. In addition Youatt (1994) has presented a compelling argu-

ment that EGTA, due to its affinity for other trace metals essential for growth, may not be an appropriate substance to use in questioning Ca^{2+} requirements for cell growth.

Calcium is absolutely required for all enzyme-activating functions of vertebrate calmodulin so far examined. However, this Ca^{2+} requirement can be changed by different concentrations of calmodulin or the presence of a calmodulin-binding protein *in vitro*. It has been shown that an increase in the calmodulin concentration can decrease the amount of Ca^{2+} required to activate calmodulin dependent enzymes and that an increase in the Ca^{2+} concentration can decrease the amount of calmodulin required for activation of calmodulin-dependent enzymes (Blumenthal and Stull, 1980). These results indicate that Ca^{2+} and calmodulin cooperatively regulate the functions of the target protein *in vitro*. However, the relationship between the calmodulin concentration in and the Ca^{2+} requirement of cells remains unclear. Transformed cells typically reveal elevated calmodulin levels as well as the ability to grow in Ca^{2+}-deficient medium, which inhibits growth of their non-transformed counterparts. However, it is difficult to determine the cause and effect relationship in this case, because it is impossible to replace the three active endogenous calmodulin genes with a single inducible calmodulin gene.

This question can be addressed in *Aspergillus nidulans* since cell growth depends on both calmodulin and Ca^{2+} concentrations and the calmodulin concentration can be experimentally manipulated. When AlcCaM cells were grown in media containing different Ca^{2+} concentrations in the presence or absence of an inducer of the AlcCaM gene, we found that increasing calmodulin levels allowed the cells to grow at very low extracellular Ca^{2+} concentrations (2 nM) (Lu et al., 1992). Even at optimal Ca^{2+} concentrations, the cells still grew faster in inducing medium than those grown in derepressing medium. Furthermore, overexpression of calmodulin decreased the requirement for extracellular Ca^{2+} by a factor of 10 (Lu et al., 1992). These studies suggest that elevating the calmodulin concentration within a cell can decrease the growth requirements for extracellular Ca^{2+}, indicating that a cooperative relationship must exist between Ca^{2+} and calmodulin inside cells. In addition, they provide a possible explanation as to why cells that are transformed and have elevated calmodulin levels, can proliferate in Ca^{2+}-deficient medium (Whitfield et al., 1980; Hickie et al., 1983; Veigl et al., 1984).

C. Requirement of Ca^{2+} and Calmodulin for Initiation of Mitosis

The mutant strains conditional for calmodulin expression have been used to determine at which points calmodulin is required during the nuclear division cycle of *Aspergillus nidulans*. We first examined the effect of reducing calmodulin levels on cell growth (Lu et al., 1992; Lu et al., 1993d). When all AlcCaM-containing strains and their respective control strains were grown in derepressing medium at the permissive temperature, all strains contained similar levels of calmodulin and were able to grow normally, as shown in the left plate of Figure 3A. However, when incubated in repressing medium at the permissive temperature, the cells containing only the *alc*A promoter-driven calmodulin gene did not grow, while their respective control strains grew normally (middle plate of Figure 3), confirming that calmodulin is an essential gene in *Aspergillus nidulans* (Rasmussen et al., 1990). Furthermore, when grown in derepressing medium at the restrictive temperature, AlcCaM/T23 and AlcCaM/A5 strains could not grow, although the AlcCaM strain did grow under the same conditions (right plate of Figure 3), indicating that the conditional calmodulin expression does not affect the nature of the temperature sensitive mutations. These results demonstrate that the AlcCaM/T23 and AlcCaM/A5 strains contain both a *alc*A promoter-regulated calmodulin gene and the temperature sensitive mutations, *nim*T23 and nimA5, respectively. We determined the terminal phenotype of the cells arrested by reduced levels of calmodulin and showed that about 85% of the nuclei were arrested in G_2 and the remaining nuclei were blocked in G_1 or S (Lu et al., 1992). Upon induction of the AlcCaM gene, the growth-arrested cells resumed germtube formation, cell growth and the nuclear division cycle (Lu et al., 1992). These results indicate that the growth-arrest caused by reduced calmodulin concentrations is fully reversible. These results also suggest that a primary calmodulin requirement exists for progression of *Aspergillus nidulans* cells into mitosis (Lu et al., 1992).

To further investigate the requirement of calmodulin for progression into mitosis, we employed the double mutants in which the AlcCaM gene was combined with either the *nim*T23 or *nim*A5 temperature sensitive mutation (Lu et al., 1993d; Lu and Means, 1993b). When the AlcCaM/T23 cells were arrested in G_2 under low calmodulin conditions (about 5% of the calmodulin present in control *nim*T23), cells were severely impaired in their ability to enter mitosis as they were released from the G_2 arrest point, when compared to the same cells containing

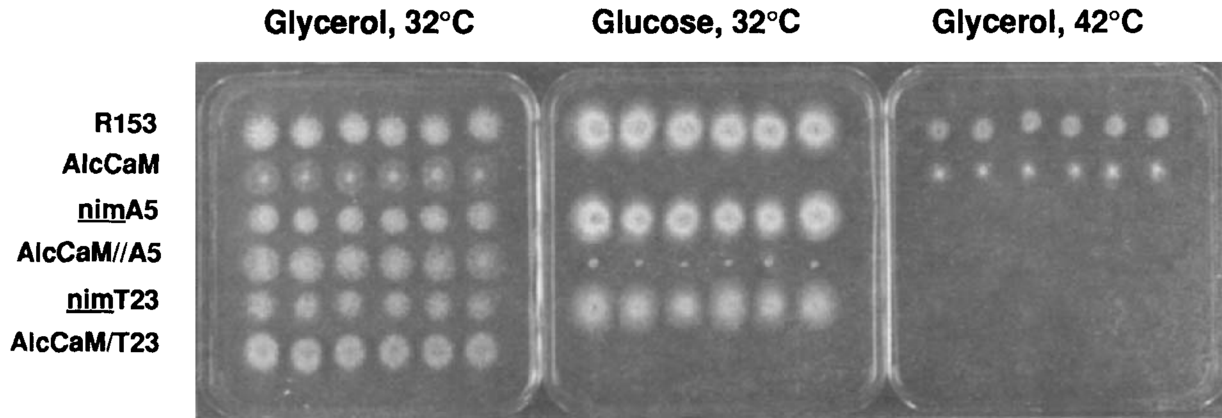
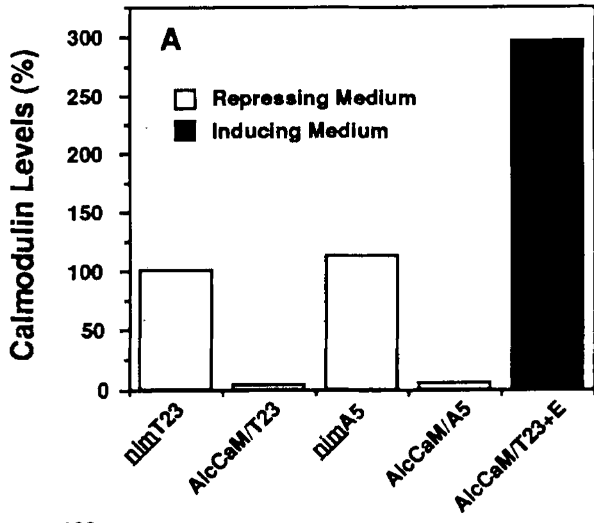


Figure 3. Evaluation of strains conditional for calmodulin expression in different genetic backgrounds.

The pAL-CaMKP vector was transfected into the nontemperature-sensitive GR5 or temperature-sensitive *nim*A5- or *nim*T23-containing strains to generate AlcCaM, AlcCaM/A5 or AlcCaM/T23, respectively. Conidia from these strains and their appropriate control strains, R153, *nim*A5 and *nim*T23 were transferred to depressing (glycerol) or repressing (glucose) plates and cell growth was assessed after 3 days incubation at 32 or 42°C.

high levels of calmodulin (about 300% of the calmodulin present in control *nim*T23) or to control *nim*T23 cells (Figure 4A and B). Following release from the G_2 arrest, over 90% of the *nim*T23 or AlcCaM/T23 cells grown in inducing medium had entered mitosis. In contrast, only 10 to 20 % of the AlcCaM/T23 cells entered mitosis after release from the G_2 block when grown in repressing media. Similar results were found when extracellular Ca^{2+} concentrations were manipulated while normal intracellular calmodulin levels were present. When the concentration of Ca^{2+} was lowered to 2 nM for only 1 hr before release of the G_2 block, cells could not execute the G_2/M transition upon return to the permissive temperature and re-addition of Ca^{2+} allowed progression into mitosis within 10 min. Reduced levels of extracellular Ca^{2+} also prevented the increase in calmodulin levels that normally occurs coincident with mitosis. These results demonstrate that both calmodulin and Ca^{2+} are required for entry into mitosis from the *nim*T23 G_2 arrest point and that Ca^{2+} may be required for synthesis of calmodulin at mitosis.

Although reduced calmodulin levels prevent entry into mitosis in the *nim*T23 genetic background, such is not the case in the *nim*A5 genetic background (Figures 4A and C). We could not detect any effect of lowered calmodulin levels on the ability of cells to enter mitosis from the *nim*A5 G_2 arrest point using the AlcCaM/A5 strain (Lu and Means, 1993b). These differences in requirements for calmodulin may be due to the possibility that the *nim*T23 and *nim*A5 mutations arrest cells at different points in G_2. This idea is supported by the observation that at the G_2 arrest point, there are fewer phosphoproteins present in *nim*T23 than in *nim*A5 cells, as detected by the MPM-2 antibody that is specific for mitotic phosphoproteins (Lu et al., 1993d; Osmani et al., 1991b). In addition, it takes 20–30 min for the *nim*T23 cells to enter mitosis from the arrest point after releasing the block relative to the *nim*A5 cells which enter mitosis in 5–10 min after release from the block (Lu et al., unpublished data). It appears that the point required for *nim*T^{cdc25} is temporally further from mitosis than is that for *nim*A. Therefore it is possible that, at the *nim*A5 arrest point, the processes that require calmodulin have already occurred so that cells could enter mitosis independent of calmodulin when the *nim*A5 mutation is released.


A
Calmodulin Levels (%)
300
250
200
150
100
50
0
Repressing Medium
Inducing Medium
nimT23
AlcCaM/T23
nimA5
AlcCaM/A5
AlcCaM/T23+E

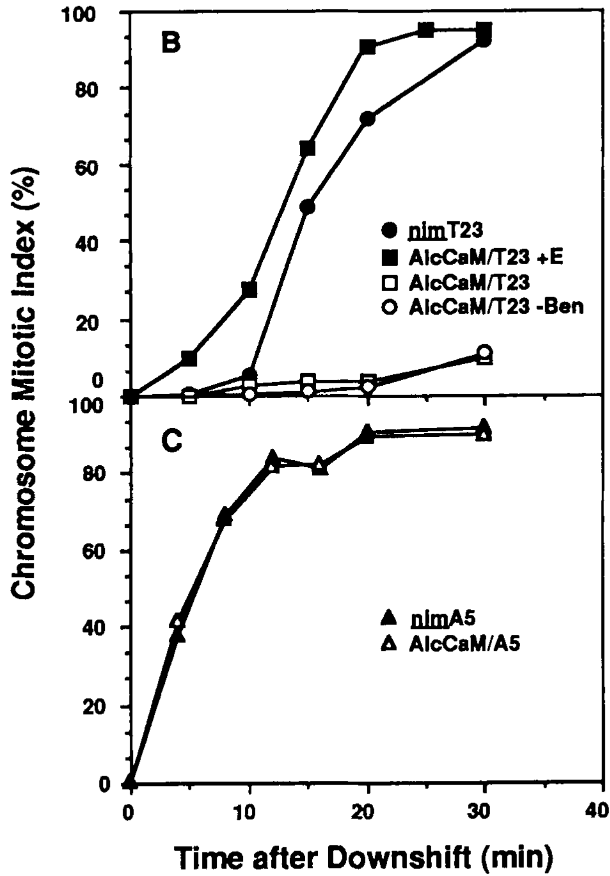

B
C
Chromosome Mitotic Index (%)
100
80
60
40
20
0
nimT23
AlcCaM/T23 +E
AlcCaM/T23
AlcCaM/T23 -Ben
nimA5
AlcCaM/A5
0
10
20
30
40
Time after Downshift (min)

Figure 4. Effect of intracellular calmodulin levels on G_2/M progression.

A. Calmodulin levels. The conidia from different strains were grown in repressing medium or inducing medium at 32°C for 4.5 hr and then at 42°C for 4.5 hr. Cells were harvested to measure calmodulin concentrations by radioimmunoassay.

B. Effect of calmodulin levels in the *nim*T23 background. The conidia from *nim*T23 and AlcCaM/T23 strains were germinated in repressing or inducing medium for 4.5 hr at 32°C and then at 42°C for 4.5 hr (time 0) to arrest cells in G_2. For the final 10 min of the 42°C incubation, benomyl was added to a final concentration of 5 µg/ml except to one culture of AlcCaM/T23. The cultures were then downshifted to 25°C for various times as indicated. Samples were harvested for determining the percentage of cells with mitotic figures. Closed circles, *nim*T23 in repressing medium; open squares, AlcCaM/T23 in repressing medium; open circles, AlcCaM/T23 in repressing medium in the absence of benomyl; closed squares, AlcCaM/T23 in inducing medium; closed triangles, *nim*A5 in repressing medium; open triangles, AlcCaM/A5 in repressing medium.

C. Effect of calmodulin levels in the *nim*A5 background. The conidia from *nim*A5 and AlcCaM/A5 strains were arrested in G_2 in repressing medium and then released from the block using condition as described in Fig. 4A. Closed triangles, *nim*A5; open triangles, AlcCaM/A5.

D. Molecular Mechanisms of Ca^{2+}/Calmodulin-Dependent Mitotic Progression

Genetic Analysis of G_2/M Progression

In the past several years, considerable progress towards an understanding of the regulation of cell proliferation has been made due to the discovery of a key regulator of the eukaryotic cell cycle, a protein threonine/serine kinase called $p34^{cdc2}$. This protein was first identified as the CDC28 gene product in *Saccharomyces cerevisiae* and later as the product of the *cdc*2 gene of *Schizosaccharomyces pombe* (Beach et al., 1982; Lee and Nurse, 1987; Murray and Kirschner, 1989b; Nurse, 1990; Lewin, 1990; Pines and Hunter, 1990; Forsbury and Nurse, 1991). The

$p34^{cdc2}$ protein kinase has been shown to associate with other proteins including mitotic cyclins and its function in regulating progression through mitosis has been found to be highly conserved among species (Arion et al., 1988; Gautier et al., 1988; Labbe et al., 1989). The activity of the $p34^{cdc2}$ protein kinase has been shown to be modulated post-transcriptionally by tyrosine and threonine phosphorylation/dephosphorylation and by interaction with cyclin proteins (for review see Murray and Kirschner, 1989b; Pines and Hunter, 1990; Forsbury and Nurse, 1991). The mitotic cyclin concentrations change during the cell cycle, increasing as cells enter the proliferative cycle, reaching a critical concentration for binding $p34^{cdc2}$ in late G_2 and then being catastrophically degraded in metaphase of mitosis (Evans et al., 1983; Swenson et al., 1986; Murray and Kirschner, 1989a; Murray et al., 1989). After cyclin binding, $p34^{cdc2}$ appears to be a target for tyrosine phosphorylation (tyrosine15 in fission yeast, Gould and Nurse, 1989; Meijer et al., 1991; Parker et al., 1991). Two protein kinases, WEE1 and MIK1, have been shown to be involved in $p34^{cdc2}$ tyrosine phosphorylation, resulting in an inactive $p34^{cdc2}$ (Featherstone and Russel, 1991; Lundgren et al., 1991). During the G_2/M transition, a phosphotyrosine phosphatase encoded by the *cdc*25 gene of *Schizosaccharomyces pombe* (and its homologs in other systems) is activated by binding to B-type cyclins (Galaktinonv and Beach, 1991) and/or protein phosphorylation (Kumagai and Dunphy, 1992; Izumi et al., 1992). This active *cdc*25 protein specifically removes the tyrosine phosphate from $p34^{cdc2}$, thereby allowing the protein kinase to become active (Gould and Nurse, 1989; Morla et al., 1989; Dunphy and Newport, 1989; Solomon et al., 1990; Dunphy and Kumagai, 1991; Gautier et al., 1991). Tyrosine dephosphorylation of $p34^{cdc2}$ has been shown to be important for G_2/M transition in human, frog and fission yeast cells, whereas such is not the case in budding yeast. In vertebrates, another important inhibitory modification of $p34^{cdc2}$ is threonine phosphorylation (Norbury et al., 1991; Krek and Nigg, 1991a; Krek and Nigg, 1991b). Threonine 14 is phosphorylated in G_2 and dephosphorylated at M. Whereas WEE1 can phosphorylate peptide substrates on threonine 14 *in vitro*. A separate kinase activity that can phosphorylate this residue has been detected in extracts of human and *Xenopus* cells (Atherton-Fessler et al., 1994; Kornbluth et al., 1994). However, the sequence and properties of this enzyme remain to be determined. Very recently Den Haese et al. (1995) have revisited the role of WEE1 in *S. pombe* and found that this enzyme can phosphorylate threonine 14 *in vivo* but that this event requires prior phosphorylation of tyrosine 15. These authors concluded

that threonine 14 phosphorylation is not required for the DNA damage or replication checkpoint controls. Thus the phosphorylation of threonine 14, the significance of this modification and the kinase or kinases involved remain appealing questions for further study. Phosphorylation of threonine167 in fission yeast (Gould et al., 1991), or threonine 161 in *Xenopus* $p34^{cdc2}$ (Solomon et al., 1992) causes an effect opposite to that produced by phosphorylation of threonine 14 and tyrosine 15. Mutations of this threonine to non-phosphorylatable residues prevent mitotic events, indicating that phosphorylation of threonine 161 is required for $p34^{cdc2}$ activity. Solomon et al. (1992) have identified an activating kinase responsible for phosphorylation of Thr161 in *Xenopus* extracts. This enzyme, termed CAK for *c*dk-*a*ctivating-*k*inase, is a multisubunit enzyme comprised of a conserved CDK-related protein kinase CDK7 (formally MO15) and cyclin H (Morgan, 1995). The CDK7/cyclin H complex itself appears to be regulated by phosphorylation. It seems that, although there is some controversy (Solomon et al., 1992), Thr161 phosphorylation may be important for $p34^{cdc2}$ to bind to mitotic cyclin (Gould et al., 1991; Norbury et al., 1991).

A homolog of *cdc*25 in *Aspergillus nidulans* has recently been identified to be the product of the *nim*T^{cdc25} gene and the two proteins are 50% identical at the amino acid sequence level (Osmani et al., 1991a; O'Connell et al., 1992). The temperature sensitive strain *nim*T23 that we have discussed previously has a mutation of *nim*T^{cdc25} and is arrested in G_2 at the restrictive temperature with $p34^{cdc2}$ tyrosine phosphorylated. Upon release from the block, $p34^{cdc2}$ kinase is tyrosine dephosphorylated and activated, resulting in entry of cells into mitosis; this suggests that both function and regulation of $p34^{cdc2}$ are conserved in *Aspergillus nidulans*. However, whereas activation of $p34^{cdc2}$ kinase is required, it is not sufficient to trigger mitosis in *Aspergillus nidulans* if the NIMA protein encoded by the *nim*A gene is not activated (Osmani et al., 1991a). Cells carrying temperature-sensitive mutations in the *nim*A gene are specifically arrested in G_2 at the restrictive temperature, but rapidly and synchronously enter mitosis when the *nim*A mutation is released by a shift to the permissive temperature (Osmani et al., 1988). Artificial overexpression of the *nim*A gene product has been shown to induce premature mitosis but cells cannot exit from this phase of the nuclear division cycle (Osmani et al., 1988). Analysis of the predicted amino acid sequence of NIMA deduced from the nucleotide sequence of the *nim*A gene suggested that NIMA belongs to the family of protein serine/threonine kinases. Antibodies specific for NIMA were used to immunoprecipi-

tate the protein from extracts of *Aspergillus nidulans* and NIMA was found to phosphorylate β-casein but not histone H1. Using a β-casein phosphorylation assay, the activity of NIMA was found to vary during the nuclear division cycle. The kinase activity is low in G_1 and S, begins to increase in G_2 and reaches a plateau in late G_2 and mitosis (Osmani et al., 1991b). These results demonstrate that NIMA is another cell cycle-regulated protein kinase required for entry into mitosis, but is different from $p34^{cdc2}$ and related protein kinases.

Calcium/Calmodulin Requirement for Activation of $p34^{cdc2}$ and NIMA

As discussed earlier in this review, when either extracellular Ca^{2+} or intracellular calmodulin levels were reduced, cells no longer entered mitosis after releasing the *nim*T23 mutation. These observations raised the possibility that Ca^{2+} and calmodulin could be involved in regulation of the activation of $p34^{cdc2}$ and/or NIMA. To test this possibility conidia from the AlcCaM/T23 and *nim*T23 strains were arrested in G_2 at the restrictive temperature, followed by a return to the permissive temperature in the presence of benomyl to allow cells to enter mitosis (Lu et al., 1993d). In the control *nim*T23 cells or the AlcCaM/T23 cells grown in inducing medium, $p34^{cdc2}$ was found to be phosphorylated on tyrosine at the restrictive temperature and dephosphorylated following release from the *nim*T23 mutation. However, when calmodulin levels were reduced in the AlcCaM/T23 cells, the level of tyrosine phosphorylation of $p34^{cdc2}$ was not affected at the G_2 arrest point but $p34^{cdc2}$ remained tyrosine phosphorylated after release from the nimT23 G_2 arrest. These results indicate that reduced calmodulin levels do not affect tyrosine phosphorylation but block tyrosine dephosphorylation of $p34^{cdc2}$. Furthermore, NIMA activity was high in *nim*T23 cells either arrested in G_2 or released into mitosis (Lu et al., 1993d). If the *nim*T23 cells were allowed to progress through mitosis from the G_2 arrest point into the next cell cycle, the elevated level of NIMA activity was significantly reduced, since progression through mitosis leads to reduction of the high mitotic levels of NIMA kinase activity (Osmani et al., 1991b). In contrast, when calmodulin levels in the AlcCaM strain were low, NIMA was neither activated at the *nim*T23 arrest point nor after release from the block. Inducing alcCaM gene expression increased the calmodulin level, activated NIMA and allowed cells to proceed into mitosis (Lu et al., 1993d). These results demonstrated that the increase in NIMA kinase activity

associated with the G_2/M period requires calmodulin. Thus, the intracellular level of calmodulin appears to be critical for mitotic activation of both $p34^{cdc2}$ and NIMA protein kinases.

Since Ca^{2+} is also required for entry into mitosis, we investigated the effects of the extracellular Ca^{2+} concentration on tyrosine dephosphorylation of $p34^{cdc2}$ and NIMA activation (Lu et al., 1993d). The *nim*T23 cells were arrested in G_2 at 42°C either under normal growth conditions or in the presence of 2 nM Ca^{2+}. The increase in NIMA activity at the *nim*T23 arrest point was not observed in the presence of 2 nM Ca^{2+}. Increasing the extracellular Ca^{2+} concentration to 1 mM allowed the normal activation of NIMA. Reduced levels of extracellular Ca^{2+} concentration did not change the state of tyrosine phosphorylation of $p34^{cdc2}$, but prevented tyrosine dephosphorylation of this enzyme by the protein phosphatase product of the *nim*T^{cdc25} gene. These data indicate that extracellular Ca^{2+}, like calmodulin, appears to be required for activation of both $p34^{cdc2}$ and NIMA protein kinases at and upon release from the *nim*T23 arrest point.

There are at least two mechanisms by which Ca^{2+}/calmodulin could be involved in activation of the two mitotic kinases. First, Ca^{2+}/calmodulin could directly interact with NIMA and/or NIMT (encoded by the *nim*T^{cdc25} gene) and serve as a regulatory subunit of the enzyme(s). Alternatively the effects could be indirect and occur via the actions of other Ca^{2+}/calmodulin-dependent protein(s) on NIMA and/or NIMT. If NIMA and/or NIMT directly interact with calmodulin, they would be expected to bind calmodulin, potentially in a Ca^{2+} dependent manner. To examine this possibility, NIMA was either immunoprecipitated from *Aspergillus nidulans* extracts, synthesized by *in vitro* transcription/translation, or expressed in and purified from *E. coli.* as a glutathione-S-transferase (GST)-NIMA fusion protein and NIMT was made by *in vitro* transcription/translation or synthesized in and purified from *E. coli.* as a GST-NIMT fusion protein. None of these NIMA or NIMT-containing preparations were able to bind detectable calmodulin, even though comparable levels of the Ca^{2+}/calmodulin-dependent protein kinase II made by *in vitro* transcription/translation, were readily detected, as assayed by the $[^{125}I]$-calmodulin overlay procedure (Lu et al., unpublished data). We also questioned whether Ca^{2+} or/and calmodulin was capable of activating the NIMA protein kinase directly *in vitro.* NIMA protein was immunoprecipitated from the AlcCaM/T23 strain grown at the restrictive temperature on repressing media or expressed in and purified from bacteria. We could not detect any significant effect of Ca^{2+}

and/or calmodulin on the β-casein kinase activity of the NIMA samples (Lu et al., unpublished data). These results suggest that the *in vivo* requirement of Ca^{2+}/calmodulin for NIMA kinase activity and tyrosine dephosphorylation of $p34^{cdc2}$ by NIMT may be indirect and therefore involve one or more Ca^{2+}/calmodulin dependent proteins as intermediates.

Specificity of the Roles for Ca^{2+} and Calmodulin in Control of the Nuclear Division Cycle

Calcium and calmodulin have been implicated in the regulation of cell proliferation for over a decade (Lu and Means, 1993a; Whitaker and Patel, 1990; Whitfield et al., 1980; Chafouleas et al., 1982; Sasaki and Hidaka, 1982). However, an important unsolved issue is whether Ca^{2+} and calmodulin affect cell cycle progression by regulating a specific control pathway or might be required for a variety of house-keeping functions, because Ca^{2+} and calmodulin have been shown to be involved in regulation of many cellular processes (Means et al., 1991). Our results obtained from studying the roles of Ca^2+ and calmodulin in the nuclear division cycle of *Aspergillus nidulans* allow us to speculate on the answer to this question. First, overexpression of calmodulin accelerates the rate of nuclear division cycle progression, whereas reduction of calmodulin levels causes cells to become arrested primarily in G_2 (Lu et al., 1992). If reduced calmodulin concentrations resulted in some general defects in house-keeping functions, cells should be arrested at multiple points in the cell cycle, with the precise number being an indication of the relative proportion of nuclei in that stage of the cell cycle. Second, in order to directly examine the specific requirement of Ca^{2+} and calmodulin for entry into mitosis, strains conditional for calmodulin expression were generated in the *nim*T23 or *nim*A5 genetic backgrounds, which may arrest cells at different points in G_2. Our results reveal that reduced calmodulin levels prevent the G_2/M transition in the *nim*T23 but not the *nim*A5 genetic background (Figures 4B and C), indicating that reduction in calmodulin does not have generally deleterious effects on cellular function. Therefore, we reasoned that reduction in calmodulin levels may specifically affect some regulatory pathway involved in the G_2/M transition.

Further biochemical analyses of these arrested cells support the notion that effects of reduced Ca^{2+} or calmodulin levels are at least selective if not specific. At the *nim*T23 arrest point, reduction of Ca^{2+} or calmodulin

levels prevents activation of NIMA but has no effect on tyrosine phosphorylation of $p34^{cdc2}$ (Lu et al., 1993d). Tyrosine phosphorylation of $p34^{cdc2}$ has been shown to be tightly regulated. A prerequisite for tyrosine phosphorylation is binding of $p34^{cdc2}$ to periodically synthesized and accumulated mitotic cyclin (Gould et al., 1991; Norbury et al., 1991). The tyrosine phosphorylation of $p34^{cdc2}$ has been shown to be carried out by protein tyrosine kinases encoded by *wee*1 and *mik*1 (Featherstone and Russel, 1991; Lundgren et al., 1991), which may be activated by serine/threonine dephosphorylation in *Xenopus* (Smythe and Newport, 1992). The mechanisms involved in tyrosine phosphorylation of $p34^{cdc2}$ have been shown to be conserved in many eukaryotic cells, presumably including *Aspergillus nidulans*. Therefore, reducing Ca^{2+} or calmodulin levels does not prevent protein synthesis, protein kinase activity or phosphatase activity in general but it does selectively prevent activation of the NIMA protein kinase at the *nim*T23 G_2 arrest point. We argue that the requirement of these molecules for activation of NIMA must be selective.

After release of the mutation in the *nim*T^{cdc25} gene, $p34^{cdc2}$ remains tyrosine phosphorylated under conditions in which the level of calmodulin or Ca^{2+} is held low. In order to examine whether any cellular processes involved in G_2/M progression take place normally under these experimental conditions, we evaluated changes in histone H1 kinase activity of proteins that are precipitated by p13 beads and mitotic protein phosphorylation detected using the monoclonal antibody MPM-2, that selectively reacts with mitotic phosphoproteins (Davis et al., 1983; Kuang et al., 1989). When control *nim*T23 cells were arrested in G_2 at the restrictive temperature, the levels of p13 associated H1 kinase activity and MPM-2-reacting proteins were low. In contrast, when the *nim*T23 mutation was released and cells entered mitosis, the H1 kinase activity and MPM-2-reacting proteins increased substantially as expected. These results suggest that when cells enter mitosis from the *nim*T23 arrest point, there are mitosis-specific increases in H1 kinase activity and protein phosphorylation. Interestingly, the increase in p13 associated H1 kinase activity and the appearance and pattern of the majority of cellular MPM-2-reacting phosphoproteins that occur after release from the G_2 arrest are not substantially changed under conditions when either calmodulin or Ca^{2+} concentrations are held low. Since p13 associated H1 kinase activity increased in the presence of tyrosine phosphorylated $p34^{cdc2}$ (Lu et al., 1993d), the kinase involved seems unlikely to be $p34^{cdc2}$. However, we cannot completely rule out this

possibility since increased H1 kinase activity associated with p34^{cdc28} has been shown to occur without apparent tyrosine dephosphorylation of p34^{cdc28} in budding yeast (Sorger and Murray, 1992; Amon et al., 1992). Another possibility is that other cdc2-like protein kinase exist on the p13 beads. In fact, we did observe another anti-PSTAIR-reacting protein with a Mr of about 37 kDa (p37) in our p13 precipitates (Lu et al., 1993d). Treatments with serine/threonine or tyrosine phosphatases did not change the ratio of p37 and p34^{cdc2}, although the tyrosine phosphatase did remove tyrosine phosphate from p34^{cdc2}. Additionally, p37 could not be detected with anti-phosphotyrosine antibodies (Lu et al., 1993d). We concluded from these studies that p37 was not a phosphorylated form of p34. Subsequently Osmani et al., (1994) cloned the gene encoding p34 and named it *nim*X^{cdc2}. Antibodies prepared to the COOH-terminus of the expressed protein precipitated both p34 and p37. Treatment of the detergent-denatured immunoprecipitate with potato acid phosphatase for 2.5 hr at 32° resulted in the disappearance of the p37 band. The authors concluded that p37 was a serine/threonine phosphate containing form of p34 but that the phosphate was not accessible to phosphatase unless the protein was denatured. However since the antibody could not detect even p34 in protein extracts or when affinity-purified using p13 beads, these experiments cannot rule out the presence of another PSTAIR protein kinase that migrates on SDS-containing gels at 37 kDa. If the increase in H1 kinase activity after release of the *nim*T23 arrest in the absence of normal levels of Ca^{2+} or calmodulin is due to a putative second PSTAIR-containing protein, then it must either be activated indirectly by NIMT or be due to the ability of NIMT to catalyze non-phosphotyrosine dephosphorylation reactions. The possibility also exists that other non-p34^{cdc2} related protein kinases could be present in the p13 precipitates as it has been recently been shown that MAP kinases can bind to this affinity matrix (Shibuya et al., 1992). Therefore, a variety of explanations could account for our observation that H1 kinase activity increases in the apparent absence of p34^{cdc2} tyrosine dephosphorylation.

In addition, our observation that the level of mitotic protein phosphorylation was not significantly modified by the absence of normal levels of Ca^{2+} or calmodulin when the *nim*T23 mutation was released indicate that the *nim*T^{cdc25} checkpoint can be passed sufficiently to allow protein phosphorylations detected by the MPM-2 antibody to occur. Since p34^{cdc2} remained tyrosine phosphorylated, it is unlikely that this kinase is directly responsible for the MPM-2 reacting phosphorylations. Although it remains possible that tyrosine phosphorylated p34^{cdc2} has

sufficient activity to produce the MPM-2 reacting phosphorylations but is insufficient to promote the modifications essential for mitosis. Interestingly Ye et al., (1995) have shown that NIMA itself can be phosphorylated by $p34^{cdc2}$ on an MPM-2 epitope but that this "hyperphosphorylation" is not required for NIMA kinase activity. Alternatively, perhaps another kinase(s) that is present on the p13 beads and activated downstream of *nim*T^{cdc25} in the absence of Ca^{2+} or calmodulin is an "MPM-2" kinase. Taken together, these data suggest that Ca^{2+} and calmodulin are required for tyrosine dephosphorylation via a mechanism involving modification of either NIMT, $p34^{cdc2}$ or the ability of these components to appropriately interact, but not due to some general inability of the cell to function correctly.

Characterization and Regulation of NIMA Protein Kinase by Serine/Threonine Phosphorylation

Since NIMA is present in very low amounts in the cell and the only assay available relies on the use of peptide specific antibodies to produce an enzymatically active immune complex, virtually nothing was known concerning its biochemical properties. In order to understand how Ca^{2+} and calmodulin may regulate NIMA, we expressed a full-length *A. nidulans nim*A cDNA in bacteria and purified NIMA as a fusion product of bacterial glutathione S-transferase (Lu et al., 1993c). The purified enzyme phosphorylates β-casein and ATP with Kms of 38 and 69 μM, respectively, and has a Vmax of 156 nmol/min/mg. Due to the abundance of purified enzyme, we were able to optimize the original assay conditions which resulted in a 100-fold increase in activity (Lu et al., 1993c). The expressed form of the enzyme will phosphorylate a number of protein and peptide substrates and, based on the latter, we suggested that Phe-Arg-Xaa-Ser/Thr represents the optimal primary sequence for NIMA kinase phosphorylation (Lu et al., 1994). Relative to exogenous substrates perhaps most interesting are the peptides that are not phosphorylated by NIMA. These include substrates that are selective for a variety of known regulatory kinases such as cyclic AMP dependent kinase, Ca^{2+}/calmodulin dependent kinase, casein kinase I, casein kinase II, MAP kinases or S6 kinases. On the other hand, a synthetic peptide substrate for protein kinase C is readily phosphorylated as are synthetic peptide analogs derived from the cardiac sarcoplamic reticulum proteins phospholemann and phospholamban (Lu et al., 1993a). This pattern of substrate preference and the unusual requirement for a Phe at the P-3

position, are considerably different from those of any other protein kinase that has been characterized to date. NIMA is also different from NPK1 and Nek1 isolated from yeast and mouse, respectively. The yeast NPK1 kinase (Jones and Rosamond, 1990; Baron et al., 1992; Schweitzer and Philippsen, 1992) is 46% identical to NIMA within the kinase homology domain but is much smaller (435 vs 799 amino acids) and encoded by a nonessential gene. Although no biochemical properties of NPK1 have been reported, it is unlikely to represent a functional homolog of NIMA. The mouse enzyme, Nek1 (Letwin et al., 1992), is 42% identical to NIMA within the kinase domain. However, biochemical properties of Nek1 have been determined and are very different from NIMA. Major differences include substrate specificities and metal ion requirements. In addition, Nek1, which was cloned from an expression library using antiphosphotyrosine antibodies, is a dual specificity kinase. It can phosphorylate IgG, histone H1 and poly(Glu:Tyr) as well as both serine/threonine and tyrosine residues in bacterial proteins. Our own results reveal NIMA to be specific for serine/threonine residues.

Schultz et al., (1994) obtained three human cDNAs related to NIMA using a PCR approach. One of these was closely related to the murine Nek1 described above. The other two were novel NIMA-related kinases and were named Nek2 and Nek3. To date, Nek 2 is the most closely related to *Aspergillus* NIMA. Both are serine/threonine specific protein kinases, show similar cell cycle dependent changes in expression patterns, are phosphoproteins and phosphorylate a similar set of proteins and synthetic peptides. However Nek2 does not require Phe at the −3 position nor has yet been found to complement *nim*A mutants of *Aspergillus nidulans* (Fry et al., 1995). So, for only functional homolog of NIMA to have been identified to date is from the related fungus *Neurospora crassa* (Pu et al., 1995). On the other hand, recent evidence suggests that a functional NIMA-related pathway must exist in cells from yeast, frogs and humans. In the effort to understand the role of NIMA in the nuclear division cycle in *Aspergillus* Osmani et al., (1988) showed that overexpression of the enzyme resulted in premature and somewhat aberrant mitotic events. This approach was extended by O'Connell et al., (1994) who showed that the COOH-terminus of NIMA results in instability of the protein. Removal of these sequences resulted in overexpression of a stable but truncated NIMA. Accumulation of the stable NIMA caused a lethal premature chromosome condensation. A similar phenotype was observed upon overproduction of this form of NIMA in *S. pombe* or human HeLa cells. A similar approach was taken by Lu and

Hunter (1995) who found that NIMA resulted in germinal vesicle breakdown in *Xenopus* oocytes and premature mitotic events in HeLa cells. Both papers showed that these lethal effects occurred without activation of $p34^{cdc2}$. Finally Pu and Osmani (1995) showed the importance of the COOH-terminus was to permit degradation of NIMA during mitosis and that such destruction was required for completion of mitosis. These remarkable properties of NIMA together with its function in cell cycle progression suggest that it may represent a member of a unique class of protein serine/threonine kinases.

Several protein kinases involved in cell cycle regulation are also subject to control by protein phosphorylation/dephosphorylation. Examples are $p34^{cdc2}$, which is phosphorylated on both tyrosine and threonine residues (Gould and Nurse, 1989; Morla et al., 1989; Dunphy and Newport, 1989; Gould et al., 1991; Norbury et al., 1991; Solomon et al., 1992) and MAP kinases which also undergo dual regulatory phosphorylation events (Rossomando et al., 1989; Anderson et al., 1990). Each of these enzymes is involved in a cascade of reactions involving a series of protein kinases and phosphatases. We therefore examined the possible role of protein phosphorylation in regulating NIMA kinase activity (Lu et al., 1993c). To address this question, we started with the recombinant NIMA, since it is possible to purify the expressed proteins and to generate a kinase negative mutant by substitution of a single Lys residue at position 40 within the ATP binding domain with Met. When bacteria containing the wild type or the kinase-negative mutant NIMA expression plasmid were labeled with [^{32}P]-phosphate, wild type but not mutant NIMA was found to be labeled with [^{32}P] and the [^{32}P] could be removed completely by incubation with protein serine/threonine phosphatase 1 (PP1). Phosphoamino acid and phosphopeptide analysis revealed that NIMA was phosphorylated exclusively on multiple serine/threonine residues. These results demonstrate that the recombinant NIMA undergoes autophosphorylation at multiple sites when expressed in bacteria. In order to determine if phosphorylation regulated NIMA kinase activity, we pretreated the recombinant NIMA with different phosphatases. Preincubations with either protein serine/threonine phosphatase 2A (PP2A) or PP1 decreased NIMA kinase activity in both a concentration- and time-dependent manner. Furthermore, okadaic acid, a potent protein serine/threonine phosphatase inhibitor, completely blocked the effect of the phosphatases at concentrations of 16.6 nM and 1 μM, respectively, when added to the reaction mixture before the phosphatases. In contrast, preincubation with protein tyrosine phosphatase 1B (PTP1B) had no

effect on the NIMA kinase activity, even at 100-fold higher concentrations than those used for the protein serine/threonine phosphatases. These results indicate that serine/threonine phosphorylation is essential for NIMA kinase activity.

When *Aspergillus nidulans* cells were arrested in G_2 by the *nim*T23 or *nim*A5 mutation, NIMA was found to be a phosphoprotein by *in vivo* [^{32}P]-phosphate labelling, followed by immunoprecipitation with the NIMA specific peptide antibodies (Lu et al., 1993c). The treatment of NIMA immunoprecipitated from the *nim*T23 G_2 arrested cells with PP2A, or PTP1B, before the kinase assays shows that PP2A, but not PTP1B, decreased NIMA activity to the basal level. Again the effect of PP2A could be prevented by the addition of 1 μM okadaic acid (Lu et al., 1993c). These results indicate that serine/threonine phosphorylation of cellular NIMA is also required for the kinase activity of this protein (Lu et al., 1993c). NIMA kinase activity has been shown to be regulated during the nuclear division cycle of *Aspergillus nidulans*, with activity being low in G_1 and S, beginning to increase in G_2 and reaching a plateau in late G_2 and mitosis (Osmani et al., 1991b). Although the changes in phosphorylation state of NIMA during this division cycle remains to be determined, NIMA is a phosphoprotein in G_2, when NIMA is normally active (Lu et al., 1993c). Ye et al., (1995) show that one enzyme that can phosphorylate NIMA is $p34^{cdc2}$. Whereas this phosphorylation exposes an MPM-2 epitope, it is not essential for NIMA kinase activity. These results indicate that NIMA phosphorylation may be an important regulatory event responsible for the change in the NIMA kinase activity during the nuclear division cycle. Our results also indicate that the *nim*A5 temperature-sensitive mutation does not affect NIMA phosphorylation, as this activating regulatory event occurs at the G_2 arrest point (Lu et al., 1993c). This may offer one explanation for the results mentioned previously that the *nim*A5 cells could enter mitosis in the presence of low Ca^{2+} and calmodulin when the *nim*A5 mutation is released.

Does the Multifunctional Ca^{2+}/Calmodulin-Dependent Protein Kinase Mediate Ca^{2+}/Calmodulin Action at the G2/M Transition?

The protein encoded by the *cdc*25 gene has been shown to be highly regulated in the cell, although it can function as a protein tyrosine phosphatase and dephosphorylate $p34^{cdc2}$ and a peptide substrate, pNPP, when expressed *in vitro* (Dunphy and Kumagai, 1991; Rasmussen, 1970; Millar et al., 1991; Lee et al., 1992; Galaktinonv and Beach, 1991).

Galaktinonv and Beach (1991) have shown that B-type cyclins associate with human cdc25A protein *in vivo* and can activate cdc25A and B protein phosphatases *in vitro.* In addition, Kumagai and Dunphy (1992) as well as Izumi et al. (1992) have independently reported that the *Xenopus* cdc25 protein undergoes an extensive phosphorylation in its NH_2-terminal region at the G_2/M transition and that this phosphorylation is important for tyrosine phosphatase activity. In mitosis $p34^{cdc2}$ and cdc25 have been reported to form a complex *in vivo* that also contains cyclin B (Gautier et al., 1991; Jessus and Beach, 1992). The consensus opinion, therefore is that cdc25 is a physiological substrate for $p34^{cdc2}$. This would create a positive feedback loop that could explain the autoamplification of MPF activity in *Xenopus* oocytes that have been injected with very small amounts of either MPF or cdc25. However, the existence of such a mechanism cannot explain the initial event that causes cdc25 to become active in G_2. This has prompted the search for another protein kinase that can phosphorylate and activate previously inactive cdc25. Izumi and Maller (1995) have detected such a protein kinase although its identity remains unknown. At least *in vitro* these authors show that one enzyme capable of activating cdc25 is the Ca^{2+}/calmodulin dependent protein kinase II (CaM kinase II). Both NIMT from *Aspergillus nidulans* and cyclin B from *Schizosaccharomyces pombe* can be phosphorylated by *Aspergillus* CaM kinase in a Ca^{2+}/calmodulin-dependent manner (Lu, K. P., Rasmussen, C. D. and Means, A. R. unpublished data), although it remains to be determined if such phosphorylations will affect the phosphatase activity of NIMT.

Supportive of the possibility that CaM kinase might play a role at the G_2/M transition are the observations that microinjection of antibodies against CaM kinase or of a synthetic peptide modeled on the autoinhibitory region of the enzyme are able to block nuclear breakdown in sea urchin eggs (Baitinger et al., 1990) whereas microinjection of a cDNA encoding a constitutive form of CaM kinase into *Xenopus* oocytes initiates maturation (Waldmann et al., 1990). It is now clear that CaM kinase II is the only target for Ca^{2+}/calmodulin required to mediate the Ca^{2+} signal responsible for the release of the meiosis II block in *Xenopus* oocytes. Lorca et al., (1993) have shown that microinjection of a Ca^{2+}/calmodulin independent form of CaM kinase IIα is sufficient to cause release from the metaphase arrest. This response does not require Ca^{2+} or calmoduin and can be prevented by the co-injection of an autoinhibitory peptide derived from CaM kinase IIα. This peptide also prevents the release from metaphase that normally results from an

increase in intracellular Ca^{2+}. Similarly, CaM kinase II is the only target for Ca^{2+} required for inducing anaphase in Cytostatic Factor (CSF) extracts that is characterized by sister chromatid separation (Morin et al., 1995). This response does not result from direct phosphorylation of metaphase spindle components. Therefore, whereas the substrates have not been identified, CaM kinase II plays a major role in the completion of meiosis. It would not be surprising if it was also the enzyme responsible for the Ca^{2+}/calmodulin requirement for mitotic progression.

Whereas we have demonstrated that NIMA undergoes autophosphorylation/autoactivation when expressed in bacteria, this does not exclude the possibility that NIMA may be regulated by other protein kinase(s) in the cell under normal circumstances. Precedence for such possibility exists for MAP kinases. Bacterially expressed MAP kinase becomes autophosphorylated on both threonine and tyrosine residues *in vitro* which activates the enzyme (Rossomando et al., 1992). Dephosphorylation of either residue results in inactivation (Anderson et al., 1990). Yet in the cell MAP kinase is phosphorylated by specific MAP kinase kinases (Matsuda et al., 1992; Nakielny et al., 1992; Kosako et al., 1992; Crews and Erikson, 1992; Rossomando et al., 1992) and the sequence of the first member of this still growing class of enzymes was reported by Crews et al. in 1992. Thus some parallels can be made as to how MAP kinase and NIMA may be activated. We have shown NIMA to be a phosphoprotein and serine/threonine phosphorylation to be required for activity both in *Aspergillus nidulans* and when expressed in bacteria (Lu et al., 1993c). In bacteria, NIMA is clearly autophosphorylated exclusively on multiple serine/threonine residues. We suspect these activating intermolecular modifications are due to the high concentration of the enzyme. When NIMA is dephosphorylated it becomes inactive. Preincubation of the enzyme under phosphorylation conditions results in a slow rate of autophosphorylation but in this case no kinase activity is recovered. Even when active NIMA is used to phosphorylate the dephosphorylated enzyme, activity of the latter is not restored (Lu et al., 1993c). However, we estimate that the enzyme concentration in the *in vitro* reaction is far lower than what exists in bacteria. Since the concentration of NIMA in *Aspergillus nidulans* appears to be very low (Osmani et al., 1991b), it is unlikely that autophosphorylation is responsible for activation of the kinase in G_2. We propose that NIMA may well also be an intermediary component in a cascade of protein kinase and phosphatase reactions that are required for progression from G_2 to mitosis (Lu et al., 1993c). This suggestion is supported by the recent findings of Ye et al., (1995) who argue that $p34^{cdc2}$ represents the last enzyme of the cascade.

To search for possible upstream protein kinases of NIMA, we questioned whether purified protein serine/threonine kinases could phosphorylate and reactivate the dephosphorylated NIMA (Lu, K. P. et al., unpublished data). Under the conditions used, CaM kinase purified from either a mammalian source or *Aspergillus nidulans*, MPF purified from *Xenopus laevis* and the catalytic subunit of cyclic AMP-dependent kinase (PKA) could phosphorylate NIMA, while MAP kinase and S6 kinase purified from mammalian cells and *Xenopus*, respectively, did not phosphorylate NIMA. Neither PKA, MPF, MAP kinase nor active NIMA could increase the kinase activity of the dephosphorylated NIMA. However, phosphorylation of the dephosphorylated GST-NIMA with mammalian CaM kinase, its *Aspergillus nidulans* homolog or a truncated monomeric form of CaM kinase (CaMK 1-317) resulted in a reproducible and statistically significant reactivation, increasing NIMA activity from 2 to 3.5 fold which represents 25–35% of the original NIMA activity. This phosphorylation and reactivation by CaM kinase depended on the presence of both Ca^{2+} and calmodulin and showed kinetics typical of known CaM kinase substrates. Furthermore, phosphorylation of NIMA by CaM kinase was sensitive to PP2A treatment but was insensitive to this phosphatase in the presence of okadaic acid. Phosphopeptide analysis revealed that NIMA phosphorylated by CaM kinase contained 4 major phosphopeptides and two of these were similar to those containing NIMA autophosphorylation sites. These results indicate that CaM kinase can phosphorylate NIMA on residue(s) that is/are susceptible to PP2A and that such phosphorylation is sufficient to partially restore NIMA kinase activity. There are several possibilities to explain why CaM kinase is unable to fully reactivate NIMA. One possibility is that NIMA requires phosphorylation by multiple protein kinases *in vivo* and CaM kinase is only one of these kinases. In this scenario, CaM kinase could be necessary but not sufficient to fully reactivate NIMA. It is also possible that NIMA requires some other protein(s) for proper folding or simply cannot fold properly *in vitro* after being phosphatase treated. If this were the case, NIMA could never be fully active after phosphatase treatment, even though all required phosphorylation sites could have been phosphorylated by CaM kinase. A third possibility is that whereas CaM kinase can activate dephosphorylated NIMA *in vitro*, it is not the physiologically relevant enzyme in the cell. To conclusively prove the role of CaM kinase phosphorylation in regulation of NIMA, it will be necessary to identify the CaM kinase phosphorylation site(s) and subsequently examine the effects of mutating these residues on NIMA functions *in vivo*.

Bartelt et al., (1988) first isolated CaM kinase from *Aspergillus nidulans* and, whereas it was found to be a monomer, showed that it was also a multifunctional enzyme with similarities to the mammalian CaM kinase II enzymes. Korstein et al., (1992) subsequently cloned the cDNA and, based on Southern analysis, suggested that CaM kinase was a unique gene. We cloned the CaM kinase gene and confirmed that it was unique by Southern, Northern, and PCR analysis. In order to examine the role of this enzyme in growth or development of *Aspergillus*, we created a strain conditional for expression of the CaM kinase gene. This was accomplished as had been previously done for calmodulin (Lu et al., 1992) by disrupting the endogenous gene by a copy of the kinase gene under the control of the alcohol dehydrogenase (*alc*A) promoter. We were surprised to find that this conditional strain grew and formed conidia on minimal media containing glucose to repress the *alc*A promoter. Repression of the CaM kinase gene had no effect on nuclear division or DNA synthesis. However cells maintained in repressing conditions exhibited slowing of cellular growth in both liquid cultures and on solid agar plates and appeared to be defective in nuclear migration. Neither overexpression of the wild type CaM kinase nor a kinase minus mutant affects growth or nuclear division. Although CaM kinase activity was not detected in extracts made from cells grown in repressing medium, CaM kinase protein and activity was recovered from these extracts following CaM-sepharose chromatography. It is possible that the subtle phenotype seen in cells grown in repressing media is due to this demonstrated leakiness of the *alc*A promoter. Thus we are attempting to disrupt the gene in both an heterokaryon and in a diploid. Furthermore, although the CaM kinase inhibitor KN-62 will inhibit the purified recombinant *Aspergillus* enzyme with a Ki of 0.6μM, there was no inhibition of cell growth on plates containing up to 20 μM KN-62. These results suggest that CaM kinase may not be essential for conidiophore formation, cell cycle progression or viability in *Aspergillus nidulans*, however final confirmation awaits the disruption of the gene.

E. An Essential Role for the Ca^{2+}/Calmodulin-Dependent Phosphatase Calcineurin in Cell Cycle Progression

Protein phosphorylation and dephosphorylation are important regulatory events in cell cycle progression. As there is one primary CaM kinase in *Aspergillus nidulans*, there appears to be only one Ca^{2+}/calmodulin-

dependent protein phosphatase as well. We have recently isolated and sequenced complete cDNA and genomic clones encoding the catalytic subunit of this Ca^{2+}/calmodulin-dependent phosphatase and the deduced protein sequence reveals that it is a homolog of calcineurin A (Rasmussen, et al., 1994). At the amino acid level, the fungus protein is 78%, 51%, and 40% identical to calcineurin A from *Neurospora crassa*, human and *Saccharomyces cerevisiae*, respectively. The calcineurin A gene is unique and disruption results in a lethal phenotype, indicating that calcineurin is essential for cell growth in *Aspergillus nidulans*. These observations are in contrast to those made in *Saccharomyces cerevisiae* in which there are 2 genes for the catalytic subunit and 1 gene for the regulatory subunit of calcineurin and none of the 3 genes are essential (Cyert et al., 1991; Liu et al., 1991; Ye and Bretscher, 1992). We also examined the levels of calcineurin mRNA during the nuclear division cycle of *Aspergillus nidulans*. The *nim*A5 cells were synchronized in G_2 by incubating at the restrictive temperature. After release from the temperature block, samples were taken at 10 min intervals to determine mitotic index and to isolate total RNA. The levels of calcineurin A and histone H2A mRNAs were determined by slot-blot hybridization using probes specific for each species. Calcineurin A mRNA increased after completion of mitosis and reached a maximum prior to the increase in histone H2A mRNA that occurs at the G_1/S boundary. When cells synchronized at mitosis with the microtubule-depolymerizing agent nocodazole were used, similar results were obtained (Rasmussen, et al., 1994). These results indicate that calcineurin expression increases prior to S phase of the nuclear division cycle, and suggested the possibility that calcineurin could be involved in progression into S phase.

A strain conditional for the expression of the calcineurin A (CnA) gene was constructed so that the only copy of the gene was under control of the *alc*A promoter. This strain grew normally in glycerol which allows a low level of expression from the *alc*A promoter and grew faster when CnA was overexpressed in threonine-containing inducing medium. However growth was arrested in a reversible fashion when cells were placed in repressing medium. This block of the nuclear division cycle occurred upon the shift of exponentially growing cells to repressing medium or upon germination of spores in repressing medium. These results confirmed the essential nature of the CnA gene. However, it has been difficult to determine the precise points of the nuclear division cycle for which CnA is required. Both exponentially growing cells and spores

contain CnA so, although in the latter cases the cells completed the first round of DNA synthesis before arresting, it is difficult to know whether the arrest observed is simply a function of when the cellular CnA becomes depleted. In an attempt to overcome this problem, we treated w^+ cells with the immunosuppressive drug FK-506. At least in yeast and mammalian cells, the primary target for FK-506 is calcineurin (Cardenas et al, 1994) and we found that *Aspergillus nidulans* cannot survive this agent. Indeed FK-506 caused similar phenotypes as observed in the conditional strain grown on repressive media. This might indicate that in spores, CnA is not required for entry into the cell cycle through the first S-phase. On the other hand we cannot be certain that spores are permeable to FK-506. If not, and a lag exists before entry occurs, the phenotype observed could again be due to the time required to effectively inhibit CnA. Thus, although we feel comfortable that CnA is essential f∂r the nuclear division cycle, we cannot be certain whether the requirement is in G_2, G_1 or both. Further experiments will be necessary to conclusively establish the arrest point and to identify essential substrates of the phosphatase.

A second phenotype results from the repression of calcineurin A for 12 hr or longer. This irreversible phenotype involved polarized vegetative growth and is also observed in the repressed conditional strain. Normally a single germ tube forms in *Aspergillus nidulans* coincidental with the completion of the first nuclear division (Doonan, 1992). The nuclei of this and subsequent divisions migrate into the germ tube at regularly spaced intervals and the germ tube continues to grow. After the third nuclear division septa form resulting in a kind of "cellularization" (Harris et al., 1994). One (or more) nucleas generally is retained between two septa and the polarized growth of each "cell" recapitulates the first by extending an ariel hyphae into which the subsequently formed daughter nuclei migrate. Repression of CnA results in precocious initiation of all these aspects of vegetative growth but in the absence of nuclear division. The three most obvious defects are: (1) formation of multiple hyphae from the germinating spore, presumably due to the disruption of bipolar cell growth, (2) precocious formation of septa in the mycelium, and (3) premature branching of the multiple hyphae that is probably secondary to the disorientation of the growth axis of the mycelium (we suspect that this is due to the disruption of orderly deposition of cytoskeletal components required for apical extension of the hyphae). The most obvious interpretation of these results is that CnA plays a role in the timing of the events required for vegetative growth that is separate

from its essential role in the nuclear division cycle. We know that the defect in polarized growth is not secondary to the nuclear division cycle arrest as a number of loss of function mutations arrest the nuclear division cycle without causing premature vegetative growth. It will be of equal interest to identify downstream targets of CnA that mediate this effect.

III. COMPARISON OF ROLES FOR CALCIUM AND CALMODULIN IN *ASPERGILLUS NIDULANS* AND *SACCHAROMYCES CEREVISIAE*

Our studies in *Aspergillus nidulans* are quite consistent with previous findings obtained in mammalian cells (for review see Lu and Means, 1993a), but differ from results obtained in *Saccharomyces cerevisiae*, as summarized in Table 1. The similarities are that in budding yeast, calmodulin is also essential for cell growth (Davis et al., 1986), intracellular calmodulin and, to some extent, Ca^{2+} concentrations change during the cell cycle (Uno et al., 1989; Nakajima-Shimada et al., 1991) and

Table 1. Comparison of Roles for the Ca^{2+}/Calmodulin Signalling System in Regulation of Growth of Vertebrate, *Saccharomyces cerevisiae* and *Aspergillus nidulans* Cells

Characteristic	*Vertebrate*	*S. cerevisiae*	*A. nidulans*
Extracellular Ca^{2+} Required for Growth	Y	N	Y
Vertebrate Enzyme-Activating Function of CaM*	Y	Y**	Y
CaM Gene Number	3	1	1
Essential Nature of CaM	Y?	Y	Y
CaM Overexpression Accelerates Growth	Y	N	Y
Number of Ca^{2+}-Binding Sites in CaM	4	3	4
Importance of Ca^{2+}-Binding for the Essential Function of CaM	?	N	Y?
CaM Kinase Gene Number	?	2	1
Essential Nature of CaM Kinase	Y?	N$	?
Calcineurin Gene Number	5#	3§	1¶
Essential Nature of Calcineurin	Y?	N	Y

Notes: CaM, calmodulin; Y, yes; N, no; Y?, indirect evidence indicating yes; ?, to be determined; *, referring to vertebrate calmodulin-dependent enzymes; **, very poorly; #, at least 3 genes for A and 2 genes for B subunit; $, two CaM kinase genes isolated and not essential; §; 2 genes for A and 1 for B subunit; ¶, one A subunit.

intracellular Ca^{2+} appears to be required for the G_2/M transition (Iida et al., 1990). One apparent difference is that whereas extracellular Ca^{2+} is required for cell growth, and overexpression of calmodulin results in a higher rate of cell growth in *Aspergillus nidulans* and mammalian cells (Means et al., 1991), this is not the case in *Saccharomyces cerevisiae*. The growth of the yeast cells neither depends on the presence of extracellular Ca^{2+} nor is affected by increasing the calmodulin level as much as 100-fold (Davis and Thorner, 1989; Iida et al., 1990). Second, *Aspergillus nidulans* and mammalian calmodulins bind 4 Ca^{2+} ions, as mentioned earlier, whereas budding yeast calmodulin only binds 3 Ca^{2+} ions (Luan et al., 1987). The fourth binding site is silent (Geiser et al., 1991) and in other calmodulins this is one of the two highest affinity sites. Third, *Saccharomyces cerevisiae* calmodulin is a poor activator of mammalian calmodulin-dependent enzymes (Luan et al., 1987; Ohya et al., 1987) in contrast to the *Aspergillus nidulans* protein which activates several mammalian calmodulin-dependent enzymes to 80–100% of maximal activity (Rasmussen et al., 1990). Furthermore, using the [^{125}I]-calmodulin overlay procedure, Ye and Bretscher (1992) found that the bovine and budding yeast calmodulins bind to the same proteins in total yeast extract, but yeast calmodulin does not recognize many mammalian proteins that are detected by the mammalian calmodulin. There are at least two genes that encode CaM kinase and three genes for calcineurin in yeast and none of these has been shown to be essential for cell growth (Ohya et al., 1991; Pausch et al., 1991; Cyert et al., 1991; Liu et al., 1991; Kuno et al., 1991; Ye and Bretscher, 1992). However, it appears that *Aspergillus nidulans* only contains a single CaM kinase gene and a single calcineurin A gene (Korstein et al., 1992; Rasmussen,, et al., 1994, respectively). Calcineurin A is essential for cell proliferation and may be required for G_1/S progression as mentioned earlier. Fourth, Sun et al. (1991) have shown that plasmids expressing either the NH_2-terminal half (Ser1-Leu76) or the COOH-terminal half (Leu85-Cys147) of calmodulin complement the growth defect of the calmodulin gene deletion when they are suitably overexpressed in budding yeast, and Persechini et al. (1991) reported that central helix deletion mutants of vertebrate calmodulin can support budding yeast cell growth when expressed at levels similar to that of wild type yeast or vertebrate calmodulin (Davis and Thorner, 1989; Ohya and Anraku, 1989). In contrast, previous studies have demonstrated *in vitro* that the two halves of vertebrate calmodulin are highly cooperative and the length of the central helix is critical for optimal function. Neither half of calmodulin is effective as an activator

of vertebrate calmodulin-dependent enzymes (Crouch and Klee, 1980; Haiech et al., 1981; Newton et al., 1985; Newton et al., 1984), although some enzymes can be activated by calmodulin fragments *in vitro* with a much lower potency, as compared with the intact protein (Kuznicki et al., 1981; Guerini et al., 1984; Wolff et al., 1986). The central helix deletion mutation results in a substantial decrease in the ability to activate calmodulin-dependent enzymes *in vitro*, as compared to the wild type protein (Persechini et al., 1989; Vanberkum et al., 1990). Finally, Ca^{2+}-binding is essential for all enzyme activating functions of calmodulin assayed *in vitro* (Means et al., 1991), but the results obtained from *Saccharomyces cerevisiae* show that various yeast or vertebrate calmodulin mutants, which either bind fewer Ca^{2+} ions or do not bind Ca^{2+} at all *in vitro*, can support cell growth at least as well as wild type calmodulin (Geiser et al., 1991). It is clear that Ca^{2+} binding is not required for calmodulin to fulfill its essential functions in budding yeast.

Calmodulin is localized at regions of cell growth in budding yeast and areas of concentration change during the cell cycle. Brockerhoff and Davis (1992) found calmodulin at the site of bud formation and followed movement into the bud, on to the tip and finally into the neck before cytokinesis. This localization was also independent of Ca^{2+} and overlapped with that of actin. Calmodulin was also identified in the shmoo tip in cells treated with α factor suggesting that this protein could be involved in polarized cell growth. These changes in localization were supported by the fact that a t^s calmodulin mutant *cmd*1-1 (created by the complementarity of two mutations as amino acids 100 and 104) revealed defects in bud emergence and growth, cytokineses and mitosis (Davis, 1992). Finally Ohya and Bottstein (1994) characterized a number of t^s yeast mutants that resulted from substitution of one or more of the 8 highly conserved phenylalanines in calmodulin with alanine. Mutations could be classified into 4 individual complementation groups: (1) actin organization, (2) calmodulin localization, (3) bud emergence, or (4) nuclear division. These results are entirely compatible with the results from the Davis laboratory and reveal multiple essential roles for calmodulin in yeast.

To date, two essential targets for calmodulin have been identified in *Saccharomyces cerevisiae* and neither requires Ca^{2+} binding for the functional interaction. The first to be identified by Geiser et al., (1993) is a protein component of the spindle pole body called Nuf 1p or Spc110p. This protein was identified both by suppressing the temperature phenotype of *cmd*1-1 and by direct protein-protein interaction in a two-hybrid

screen. The calmodulin binding portion of Spc110p was localized to the COOH-terminal region and the requirement for calmoduin for mitosis *in vivo* could be eliminated by removal of the calmodulin binding segment. The second essential calmodulin target to be identified is the unconvential myosin, Myo2p (Brockerhoff et al., 1994). A number of lines of evidence was used to reach this conclusion including a synthetic lethal genetic screen between mutants conditional for *myo2* and *cmd1*. These findings demonstrate that Myo2p is a calmodulin target at sites of cell growth. Undoubtedly more essential calmodulin binding proteins will be identified before long. An inescapable conclusion from the yeast studies is that other organisms seem certain to have similar essential targets for calmoduin that are likely to be independent of Ca^{2+} binding. It is not surprising that other organisms could have evolved essential Ca^{2+} dependent targets for calmoduin (such as CnA in *Aspergillus*) but it is not likely that essential functions in yeast would have disappeared entirely from other species.

The independence of cell growth on extracellular Ca^{2+} in budding yeast may be due to the fact that there is a large vacuole in these cells that is filled with Ca^{2+} (Ohsumi and Anraku, 1983). This idea is supported by the fact that depletion of intracellular Ca^{2+} prevents cell growth (Iida et al., 1990). However even though Ca^{2+} is required for viability, calmodulin can clearly perform its essential function without Ca^{2+}-binding (Geiser et al., 1991). One explanation for this paradox has been offered by Rose and Vallen (1991), who suggested that the essential function of Ca^{2+} could be carried out by other yeast calmodulin-like protein(s), such as *CDC*31 which is required for duplication of the microtubule organizing center (Baum et al., 1986) and is also a component of the spindle pole body (Spang et al., 1993). Determining whether the essential function of *CDC*31 requires Ca^{2+}-binding will be required to evaluate this possibility. Another possibility is that because of the high intracellular Ca^{2+} concentration, yeast has evolved regulatory mechanisms that are independent of Ca^{2+}. Indeed, when the intracellular stores of calcium are depleted, cell cycle dependent requirements for Ca^{2+} are indicated by a transient block in G_1 followed by a stable block in G_2 (Anraku et al., 1991).

IV. SUMMARY AND PROSPECTS

Calcium and calmodulin have been widely implicated in regulating the cell cycle in vertebrate cells. However, because of the multiplicity of the

calmodulin genes and their cellular functions, it is difficult to determine whether Ca^{2+} and calmodulin are specifically involved in a regulatory pathway that controls a particular transition of the cell cycle in mammalian cells. To address these questions, we chose the genetically tractable *Aspergillus nidulans* as our model system. *Aspergillus nidulans* contains a unique and essential calmodulin gene encoding a protein product that is highly conserved both structurally and functionally, when compared to vertebrate calmodulin. By generating and studying strains conditional for the expression of calmodulin in different genetic backgrounds of *Aspergillus nidulans*, we were able to show that both the intracellular calmodulin and extracellular Ca^{2+} concentrations are important and cooperative factors in regulating the nuclear division cycle. These are in contrast to the results obtained from *Saccharomyces cerevisiae* that cell proliferation depends on the concentration of neither intracellular calmodulin nor extracellular Ca^{2+}. Our results have further demonstrated a selective requirement of Ca^{2+} and calmodulin for the initiation of mitosis and also identified two critical mitotic kinases, $p34^{cdc2}$ and NIMA, as potential molecular targets for their action at this transition point of the division cycle. Finally we have shown that the protein phosphatase calcineurin is one essential target for Ca^{2+}/calmodulin relative to control of the nuclear division cycle.

To further investigate the regulatory mechanism of NIMA by Ca^{2+} and calmodulin, the NIMA kinase was expressed in and purified from bacteria. Characterization of the enzyme led to the development of a new assay which increased kinase activity by a factor of 100 and revealed NIMA to be a charter member of a new family of protein serine/threonine kinases. We have identified a new regulatory mechanism for NIMA involving reversible phosphorylation on serine/threonine residues. Our studies also suggest that Ca^{2+}/calmodulin may be required for activation of NIMA and $p34^{cdc2}$ via a pathway involving a cascade of protein phosphorylation/dephosphorylation reactions involved in the G_2/M transition. A hypothetical scheme of such events is presented as Figure 5. As cells progress through S and G_2, mitotic cyclin is synthesized and accumulated. As the cyclin concentration increases, it binds to $p34^{cdc2}$ and this binding may also require $p34^{cdc2}$ to be phosphorylated on threonine167 by a yet to be characterized protein kinase. This cyclin binding targets $p34^{cdc2}$ for phosphorylation on tyrosine15 by the tyrosine protein kinases, WEE1 and/or MIK1, which must be activated by yet to be identified protein serine/threonine phosphatase(s). The resulting tyrosine phosphorylation keeps $p34^{cdc2}$ in an inactive state, preventing

premature entry into mitosis. These processes do not require Ca^{2+} or calmodulin. At the G_2/M transition, the intracellular free Ca^{2+} concentration is increased transiently and is presumably released from intracellular Ca^{2+} pools as reducing extracellular Ca^{2+} for 10 min has no effect on

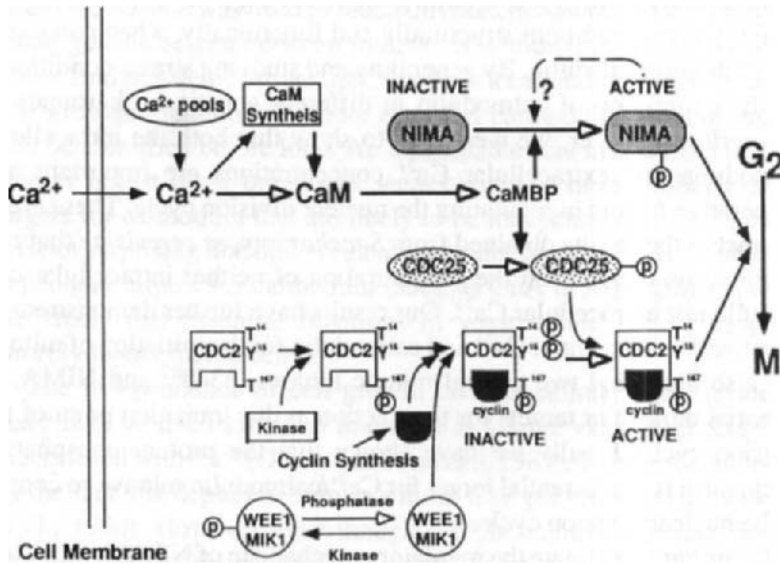


Figure 5. Regulation of mitotic kinases and potential molecular mechanisms of Ca^{2+} and calmodulin actions at the initiation of mitosis.

The activity of the $p34^{cdc2}$ protein kinase is regulated post-transcriptionally by interaction with cyclin proteins and tyrosine and threonine phosphorylation/dephosphorylation, while the activities of cdc25 and NIMA are altered by serine/threonine phosphorylation. At the G_2/M transition, a transient increase in intracellular free Ca^{2+}, released from intracellular Ca^{2+} pools, acts a primary signal for the onset of mitosis, including the selective synthesis of calmodulin. Ca^{2+} binds to calmodulin and then activates some calmodulin-binding protein(s) (CaMBP). The CaMBP regulates NIMA and/or cdc25 (NIMT), leading to activation of both $p34^{cdc2}$ and NIMA protein kinases. Open arrows indicate critical activating steps by other regulatory factors while the broken line indicates that NIMA could be autophosphorylated/autoactivated when overexpressed at high levels.

entry into mitosis. The Ca^{2+} transients trigger the synthesis of calmodulin (probably at a post-transcriptional level), which amplifies the Ca^{2+}/calmodulin responses. The resulting Ca^{2+}/calmodulin complex activates some calmodulin-regulated protein(s) (CaMBP), the most likely candidates being calcineurin and/or CaM kinase. The CaMBP then participates in a cascade of events that result in activation of NIMA protein kinase as well as NIMT (cdc25 protein), a tyrosine phosphatase that dephosphorylates and activates $p34^{cdc2}$. When both NIMA and $p34^{cdc2}$ are activated, cells will progress from G_2 into M. Now that we have generated strains that are conditional for expression of calcineurin or CaM kinase in different genetic backgrounds of *Aspergillus nidulans*, it should be possible to determine where each protein fits into the hypothetical scheme depicted in Figure 5.

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CELL CYCLE CONTROL BY PROTEIN PHOSPHATASE GENES

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ABSTRACT

The importance of protein phosphatases in eukaryotic cell cycle control was only recently established. Mutations in both protein tyrosine and serine/threonine phosphatase genes display distinct cell cycle phenotypes. Classes of phosphatases identified in fission yeast are described in regard to their positive and negative roles in the regulation of cell division cycle. Type 2A-like ppa2 and type 1-like dis2 phosphatases play vital roles in the regulation of mitotic entry and exit. The negative regulation of mitotic entry by ppa2 phosphatase, which is the *in vivo* target of okadaic acid, is discussed. An intermediate class phosphatase ppe1 controls cell shape and is implicated in mitotic control. A phosphatase regulator, sds22, which directly interacts with the dis2 catalytic subunit and controls the midmitotic progression, is identified and its role in mitosis discussed.

I. INTRODUCTION

Protein phosphorylation and dephosphorylation are important posttranslational modifications required for a variety of biological regulatory systems. In contrast to the wealth of information on the role of protein kinases in cell division and growth, some of which are known to be the product of protooncogenes, our knowledge of the biological functions of protein phosphatases is limited, particularly about the ways in which they are involved in cell division cycle control. Protein phosphatases were once thought to be enzymes that served merely to counteract the action of the protein kinases. However, as the complex regulatory nature of phosphatases is becoming apparent, protein phosphates are now known as more than simple on–off switches of the target proteins. Certain cell cycle steps are controlled by protein dephosphorylation rather than by phosphorylation.

The known number of kinases is ever increasing, but only a restricted number of phosphatases are documented. Protein phosphatases in eukaryotic cells fall into two major classes, namely, serine/threonine phosphatases and tyrosine phosphatases. In mammals there are generally four serine/threonine (ser/thr) phosphatases, that is, type 1 (abbreviated PP1), 2A (PP2A), 2B (PP2B), and 2C (PP2C), while protein tyrosine phosphatases (PTPs) constitute a gene family with and without transmembrane domains [Cohen, 1989; Fischer et al., 1991]. This article will focus on aspects originating from studies, particularly of genetic approaches, on the essential role of protein ser/thr phosphatase genes in fission yeast cell division cycle control.

II. INITIAL IDENTIFICATION OF PROTEIN PHOSPHATASE MUTATIONS

Mutations in ser/thr phosphatase genes that produce defects in nuclear division were identified in two lower eukaryotes, *Aspergillus nidulans* [Doonan and Morris, 1989] and the fission yeast *Schizosaccharomyces pombe* [Ohkura et al., 1989]. *A. nidulans BimG* and *S. pombe dis2* mutants fail to complete mitosis. Gene cloning by complementation of the mutant phenotype demonstrated that protein products encoded by *BimG* and *dis2*$^+$ genes, respectively, are approximately 80% identical in amino acid sequences to the catalytic subunit of mammalian PP1 [Doonan and Morris, 1989; Ohkura et al., 1989]. *Drosophila* mutants in one of the four PP1 isozymes also display mitotic defects [Axton et al., 1990]. The budding yeast *Saccharomyces cerevisiae sit4* mutant initially identified to be defective in transcriptional initiation of the *HIS4* gene was found to encode the catalytic subunit of a phosphatase, 43% and 60% identical to mammalian PP1 and PP2A, respectively [Arndt et al., 1989].

In the eukaryotic cell cycle, the cdc2 protein kinase plays a pivotal role as a component of mitosis promoting factor, MPF [e.g., Nurse, 1990], that is, its phosphorylation state changes during the cell cycle and its activity is regulated by phosphorylation/dephosphorylation of cdc2 polypeptide [Gould and Nurse, 1989]. The cdc2 kinase is both tyr and ser/thr phosphorylated. The requirement of tyr dephosphorylation for cdc2 activation appeared to implicate a tyr phosphatase in the control of cdc2 activation. The *cdc25*$^+$ was shown to code for the tyr phosphatase and to directly dephosphorylate the Tyr-15 residue of cdc2 polypeptide [Dunphy and Kumagai, 1991; Gautier et al., 1991; Millar et al., 1991].

III. TYPE 1 AND TYPE 2A-LIKE PROTEIN SER/THR PHOSPHATASE GENES ESSENTIAL FOR CELL DIVISION

The fission yeast cold-sensitive (cs) mutation *dis2-11* is semidominant. Cells with the gene disruption of *dis2*$^+$ are viable and grow normally, thus indicating that *dis2*$^+$ is nonessential for viability [Ohkura et al., 1989]. This is also true for *Aspergillus BimG* [Doonan and Morris, 1989]. In fission yeast the second phosphatase gene *sds21*$^+$, the protein product of which is highly similar to dis2 protein (~80% identical in amino acid sequence), could complement *dis2-11* when introduced by multicopy plasmid carrying the *sds21*$^+$ gene [Ohkura et al., 1989]. These two *dis2*$^+$

and *sds21*⁺ genes appear to have an overlapping essential function because cells with the double gene disruption are lethal (Figure 1). Spores with double gene disruption (designated Δ*dis2*, Δ*sds21*) did not germinate. In *S. cerevisiae* there seems to exist only one dis2-like phosphatase, and disruption of the gene *DIS2S1* (also called *GLC7*) causes cell death [Clotet et al., 1991; Sneddon et al., 1990]. Phosphatase

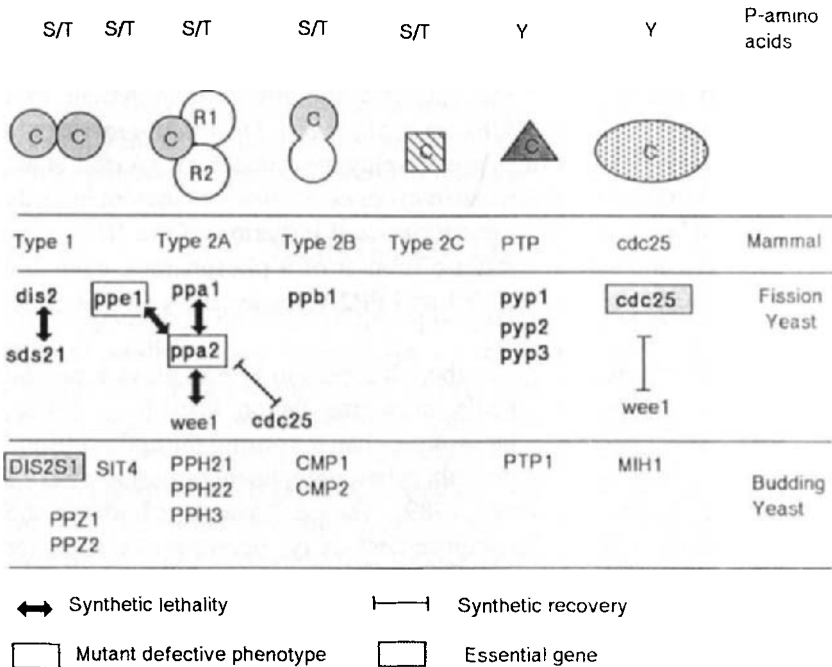

Figure 1. Protein phosphatase genes in mammals and yeasts. S/T and Y, respectively, designate serine/threonine and tyrosine residues (indicated by P-amino acids) that are dephosphorylated by phosphatases. C and R represent, respectively, catalytic and regulatory subunits. The catalytic subunits of type 1, 2A, and 2B phosphatases have similar amino acid sequences. PTP, protein tyrosine phosphatases. The thick lines with the arrowheads at both ends indicate the synthetic lethality when multiple mutants are made. The thin line with the bar at both ends indicates the synthetic recovery (suppression) when double mutants are made. Mutants with defective phenotypes are isolated from the boxed genes. The genes with the hatched box are lethal upon gene disruption.

activity estimation of yeast cell extracts indicated that dis2 and sds21 represented the major and minor phosphatases respectively, similar to mammalian PP1 [Kinoshita et al., 1990]. The type 1-like phosphatase activity in *dis2-11* is low. At the restrictive temperature the activity was negligible. It is not understood whether the activity of sds21 phosphatase is inhibited by cs dis2 protein.

Fission yeast has two PP2A-like phosphatase genes (*ppa1*$^+$ and *ppa2*$^+$; Kinoshita et al., 1990) that encode polypeptides approximately 80% identical to mammalian PP2A. Similar to the PP1-like genes, single gene disruptants are viable but the double gene disruption is lethal, thus making it appear that these two genes share an essential function for viability (Figure 1). A cs *ppa2* mutant was made by site-directed mutagenesis, which causes a dominant-lethal phenotype when introduced into cells by multicopy plasmid [Kinoshita et al., 1990]. Similarly in budding yeast, two PP2A-like genes (*PPH21* and *PPH22*) were isolated, and their double gene disruption was led to cell death [Sneddon et al., 1990]. A third PP2A-like gene (*PPH3*) was found in budding yeast [Ronne et al., 1991].

IV. OTHER PROTEIN SER/THR PHOSPHATASE GENES IN YEASTS

So far seven protein ser/thr phosphatase genes have been identified in fission yeast (Table 1). Nine protein ser/thr phosphatase genes are found in *S. cerevisiae* (Figure 1). The product of the *S. pombe ppe1*$^+$ gene [Shimanuki et al., 1993] is approximately 40%, 50%, and 70% identical to dis2, ppa2, and the budding yeast SIT4 phosphatases, respectively. It is also similar to *Drosophila* PPV (68%) and rabbit PPX (61%) phosphatases. A gene disruption (designated Δ*ppe1*) caused cs lethality and short, pear-shaped cells. These phenotypes were suppressed by four classes of multicopy suppressor genes encoding (1) ppa1 and ppa2 phosphatases, (2) mitotically essential 110-kDa dis3 protein [Kinoshita et al., 1991a], (3) protein kinase C-like pck1 [Toda et al., 1993], and (4) the budding yeast SIT4 phosphatase [Sutton et al., 1991]. Phosphatase ppe1 may play a role in cell morphogenesis and mitosis by either regulating or being regulated by these multicopy suppressor gene products. Consistent with this hypothesis, double mutants *ppe1-ppa2* and *ppe1-pck1* are lethal at the permissive temperature. The gene *ppe2*$^+$ encoding another intermediate phosphatase has been isolated from fission yeast [Shimanuki, M., unpublished results]. Other types of interme-

Table 1. Protein Serine/Threonine Phosphatase Genes Identified in Fission Yeast

Genes	*Type*	*Mutants*	*Phenotype of gene disruptant*
dis2^{+}	1	cs, del	Viable*
sds21^{+}	1	del	Viable
ppa1^{+}	2A	del	Viable#
ppa2^{+}	2A	cs, del	Viable
ppb1^{+}	2B	del	Viable
ppe1^{+}	im	del^{cs}	cs
ppe2^{+}	im	del	ND

Notes: im: intermediate
*Lethal when both *sds21*$^{+}$ or *dis2*$^{+}$ were disrupted.
#Lethal when both *ppa1*$^{+}$ or *ppa2*$^{+}$ were disrupted.
ND: not determined

diate phosphatase genes *PPZ1* and *PPZ2* (60% identity to PP1 and 40% identity to PP2A) have been found [Da Cruz e Silva et al., 1991] in *S. cerevisiae*.

The genes similar to mammalian calcineurin (PP2B) have been found in yeasts. In *S. cerevisiae*, two genes *CMP1* and *CMP2* are present [Liu et al., 1991; Cyert et al., 1991]. The degree of identity between predicted *CMP1* and mammalian CNAa proteins is approximately 50%. The disruption of single and double genes indicates that these genes are not essential for normal cell division. A single gene (*ppb1*$^{+}$) encoding a PP2B-like phosphatase has been isolated [Yoshida et al., 1994] in *S. pombe*. The PP2C-like activities have been detected in *S. cerevisiae* and *S. pombe* extracts [Cohen et al., 1989; Kinoshita et al., 1990]. A PP2C-like gene has been identified in fission yeast [Shiozaki and Russell, 1995].

V. PHENOTYPES OF *DIS2-11* PHOSPHATASE MUTANTS

In contrast to the narrow substrate specificity of cdc25 tyr phosphatase, PP1- and PP2A-like phosphatases have broad substrate specificities [Cohen, 1989]. Pleiotropic phenotypes are thus expected for mutants defective in dis2/sds21 and ppa2/ppa1 ser/thr phosphatases. Defective

phenotypes actually observed in mutants for these phosphatases, however, are surprisingly specific. The failure of sister chromatid separation in the presence of chromosome condensation and the spindle formation was found in the cs *dis2-11* mutant [Ohkura et al., 1988 and 1989]. Histone H1 kinase activity directed by cdc2 kinase was high in the mutant cells [Kinoshita et al., 1991b], whereas histone H1 phosphatase was reduced [Kinoshita et al., 1993], thus indicating that the cs *dis2-11* cells entered mitosis but failed to exit from it. A similar phenotype was found when *dis2-11* was incubated at the restrictive temperature in the presence of hydroxyurea. Hence mitotic events found in *dis2-11* could prematurely occur in the absence of DNA replication [Yamano et al., unpublished result]. The implication of this unexpected result will be discussed elsewhere. We investigated the phenotypes produced by the overexpression of *sds21*$^+$ and *dis2*$^+$ phosphatases and found that they are not identical [Yamano et al., 1994].

If a multicopy plasmid carrying the *bws1*$^+$ gene (identical to *dis2*$^+$) was introduced into the *wee1-cdc25* double mutant, which was able to grow at the restrictive temperature, the transformant was reversed to the temperature-sensitive species [the *bws* stands for *b*ypass *we*e1 suppression; Booher and Beach, 1989]. The mechanism of this reversal is unknown. By truncating and overexpressing the *dis2*$^+$ gene in *wee1-cdc25* cells, we found that the full-length dis2 polypeptide is not needed for this *bws* suppression, only a small portion of it being sufficient [Yamano et al., unpublished result].

VI. PHENOTYPES OF *PPA2* MUTANTS

PP2A-like *ppa2* phosphatase mutants displayed a phenotype distinct from dis2/sds21 mutants. To understand their role in the cell division cycle, we investigated deletion or overexpression mutant phenotypes of the *ppa1*$^+$ and *ppa2*$^+$ phosphatase genes and the *in vivo* effect of okadaic acid, an inhibitor for protein ser/thr phosphatases, on cell division [Kinoshita et al., 1993]. The *ppa2*$^+$ gene was shown to genetically interact with cell cycle regulators *cdc25*$^+$ and *wee1*$^+$; *ppa2* deletion is lethal when combined with *wee1-50*, but partially suppresses *cdc25-22*. The *ppa2*$^+$ gene appears to negatively control the entry into mitosis, possibly through the regulation of cdc2 tyrosine phosphorylation. The ppa2 phosphatase is abundant in the cytoplasm, in contrast to the nuclear localization of dis2 [Ohkura et al., 1989]. Overproduced ppa1 or ppa2 accumulates in the cytoplasm near the nuclear periphery, and cells are

arrested in interphase. Okadaic acid-treated cells, like *ppa2* deletion [Kinoshita et al., 1990], are short and display protein hyperphosphorylation. The genetic locus determining okadaic acid sensitivity in fission yeast is *ppa2*. The *ppa2* deletion reveals the same hyperphosphorylated proteins as okadaic acid. When *ppa2* deletion was treated with okadaic acid, cell size was further reduced to that of *wee1-50* or overexpressed *cdc25*$^{+}$. These results suggested a functional relationship between ppa2 and cdc25 and/or wee1 kinase.

VII. A CELL CYCLE RELATED PHOSPHATASE REGULATOR, SDS22

The fission yeast *sds22*$^{+}$ gene was initially isolated as a high gene dosage suppressor for cs *dis2-11* [Ohkura and Yanagida, 1991]. The *sds22*$^{+}$ product is a relatively small protein, having 11 tandemly arranged leucine-rich 22 amino acid repeat units in the central region [Stone et al., 1993]. Disruption of the *sds22*$^{+}$ gene was lethal, showing that it is essential for viability. Cells of Δ*sds*22 deletion displayed the characteristic mitotic phenotype, that is, cells arresting at the stage similar to metaphase (Ohkura et al., 1991). The *sds22*$^{+}$ gene thus appears to be required for exit from the metaphase.

By immunoprecipitation, sds22 protein was shown to be physically bound to the dis2 and sds21 proteins and the resultant sds22-associated phosphatase activity was shown to have altered substrate specificity [Stone et al., 1993]; this complex has the activity toward histone H1 but not phosphorylase. The loss of sds22 function by a temperature-sensitive mutation leads to cell cycle arrest at midmitosis when cdc2-dependent histone H1 kinase activity is high and sds22-dependent H1 phosphatase activity is low. To examine the unusual properties of sds22 protein structure, a collection of *sds22* deletion and point mutants were analyzed by a variety of functional criteria. It was proposed that sds22 is a regulatory subunit of the dis2/sds21 phosphatase catalytic subunits and that sds22-bound phosphatase carries a key phosphatase activity essential for progression from metaphase to anaphase (Stone et al., 1993). Mutational analysis indicates that dis2/sds21 interacts with the central repetitive domain of sds22, while the C-terminal and central regions are involved in subcellular targeting and the N-terminus is important for stability.

VIII. ROLES OF PROTEIN PHOSPHATASES PPA1/PPA2 AND PPE1 IN CELL CYCLE CONTROL

Okadaic acid causes pleiotropic abnormalities such as cell-length decrease, accumulation of binucleated cells, and protein hyperphosphorylation in fission yeast (Kinoshita et al., 1993). Single deletion mutant Δ*ppa2* is hypersensitive to okadaic acid. In this mutant, cell size reduction and hyperphosphorylation was observed without okadaic acid but the defect in cytokinesis was not. The deletion of additional *ppa1*$^{+}$ may be needed for producing such a phenotype. Alternatively, other phosphatase(s) sensitive to okadaic acid might be responsible for the defect in cytokinesis. Type 2A-like phosphatases thus appear to play a role in genetic control of cell length and cytokinesis. This agrees with the cytoplasmic localization of ppa2 phosphatase.

The genetic interaction of *ppa2*$^{+}$ with wee1^{+} and/or *cdc25*$^{+}$ is supported by the following evidence. The double mutation *wee1-50* Δ*ppa2* causes synthetic lethality, while the ts phenotype of *cdc25* is partly suppressed by the loss of *ppa2*$^{+}$. The semi-*wee* phenotype was produced in Δ*ppa2* cells and the *true wee* phenotype in Δ*ppa2* cells treated with okadaic acid. No biochemical data, however, have been obtained, which indicates direct interactions among ppa2 phosphatase, wee1 kinase, and cdc25 tyr phosphatase. Three *cdc2* alleles have been examined, but none of them showed any genetic interaction with *ppa2* mutation. It was therefore suggested that ppa2 might regulate tyrosine phosphorylation in cdc2 kinase rather than the direct dephosphorylation of cdc2 kinase (Yanagida et al., 1992; Kinoshita et al., 1993). These results strongly support the negative role of ppa2 (and also ppa1) in the entry into mitosis. The interphase arrest by the overexpression of *ppa1*$^{+}$ and *ppa2*$^{+}$ (Kinoshita et al., 1993) is consistent with the above postulated role of these phosphatases in the mitotic entry.

It remains to be determined which gene, *wee1*$^{+}$ or *cdc25*$^{+}$, is more closely related to *ppa2*$^{+}$. The ppa2 phosphatase either directly inactivates cdc25 or activates wee1 by dephosphorylation. Note that double mutation in *wee1-50* and one of the following three, namely, *mik1* deletion (Lundgren et al., 1991), multiple copies of *cdc25*$^{+}$ (*OPcdc25*; Russell and Nurse, 1987), or a *cdc2 wee* allele (Russell and Nurse, 1987) leads to mitotic catastrophe. Double mutant *wee1-50 -ppa2* is also lethal, but the catastrophic phenotype was not found (Kinoshita et al., 1993). If *ppa2*$^{+}$ is equivalent to *mik1*$^{+}$, the same catastrophe phenotype should have been observed in *wee1*-Δ*ppa2*, but it was actually not found

(unpublished result). If cdc25 is activated in *Δppa2* cells, then the lethality of *wee1 Δppa2* might be similarly reasoned to be that of *wee1-50* overproducing *OPcdc25* (Russell and Nurse, 1986 and 1987). The activation of cdc25 phosphatase would explain the ability of *Δppa2* mutant to partly suppress *cdc25-22*. A hypothesis that cdc25 activation is directly controlled by ppa2 phosphatase is therefore possible. Our results are also consistent with the hypothesis that ppa2 directly activates wee1 kinase.

The ppe1 phosphatase possesses a cellular function different among phosphatases found in fission yeast; *Δppe1* cells are aberrant in cell shape (Shimanuki et al., 1993). Because this cell morphology phenotype has not been found in other phosphatase mutants identified in fission yeast, but is seen even in growing *Δppe1* cells at the permissive temperature, this phosphatase gene function appears to be implicated in the control of cell shape. The mechanism of governing how ppe1 phosphatase actually participates in forming the rod-shaped cell morphology, however, is poorly understood. The loss of *ppe1⁺* is solely responsible for the cell-shape phenotype because plasmid carrying the *ppe1⁺* gene fully complements the phenotype.

The cell length of *Δppe1* is noticeably reduced, comparable to that of *wee1-50* mutant. However, *Δppe1* was distinct from *wee1-50* because its cell width was large; therefore, the total cell volume of *Δppe1* at the restrictive temperature is roughly the same as that of wild type, whereas the cell volume of *wee1-50* is approximately half of wild type. The short-cell-length phenotype of *Δppe1* is thus not considered to be the same as that of *wee* mutants. The cell length of *Δppe1* is shorter at 26°C than at 33°C so that a correlation might exist between cs lethal phenotype and cell length shortening. The lack of ppe1 phosphatase may produce aberration in cytoskeletal structures and cause a gross change in cell shape.

Three types of multicopy suppressor genes, namely, phosphatases ppa1 and ppa2, mitotically required dis3 protein, and PKC-related kinase pck1, have been found. In addition to these, Matsumoto and Beach (1993) show that mutations of the fission yeast *pim1⁺* gene, which is similar to human *RCC1* (Matsumoto and Beach, 1991), were suppressed by *Δppe1* function. These results established that ppe1 phosphatase has pleiotropic functions. Because the double mutant *ppa2 ppe1* is lethal, it shares the essential function for viability. Substrate proteins related to cell-length control might be regulated by both ppe1 and ppa2 phosphatases.

Multicopy plasmid carrying the *dis3⁺* gene suppresses the *Δppe1* phenotype. The *dis3* mutation is defective in mitotic chromosome disjunction, and the *dis3⁺* gene encodes a 110-kDa protein essential for viability (Kinoshita et al., 1991a). The *dis3⁺* gene is related to *dis2⁺*, because the double mutant *dis2-dis3* is lethal. The connection between *dis3⁺* and *ppe1⁺* was not unexpected, since the budding yeast *sit4* mutations were suppressed by *SSD1/SRK1* similar to *dis3⁺* (Kinoshita et al., 1991a). The implication of ppe1 phosphatase in mitosis is not well understood.

The phenotypic similarity between *Δppe1* and *Δpck2* in producing pear-shaped cells strengthens the link between ppe1 phosphatase and pck1 kinase (Toda et al., 1993). Furthermore, the synthetic lethality of the double mutant *Δppe1 Δpck1* gene disruptants suggest that these genes share an essential function. The phosphatase ppe1 might thus directly interact with pck1 kinase or indirectly upregulate the activity of pck1 kinase. The fact that *Δppe1* is hypersensitive to staurosporine is consistent with this notion. Alternatively, pck1 kinase may upregulate ppe1 phosphatase. These two genes appear to be closely related in cell-shape control.

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THE cdc25 PHOSPHATASE: BIOCHEMISTRY AND REGULATION IN THE EUKARYOTIC CELL CYCLE

Ingrid Hoffmann, Paul R. Clarke, and Giulio Draetta

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ABSTRACT

Cdc25, a universally conserved M-phase inducer, is a protein phosphatase that directly dephosphorylates and activates the cdc2/cyclin B protein

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kinase. Recently it has been found that phosphorylation regulates the activity of cdc25 during the cell cycle. The activation of cdc25 can be mediated by cdc2/cyclin B itself thus creating a positive feedback loop. A type-2A phosphatase can specifically dephosphorylate and activate cdc25. In this article we shall review our current knowledge about the regulation of entry into mitosis by the cdc25 phosphatase and its involvement in the self-amplification of MPF *in vivo*.

I. INTRODUCTION

In eukaryotic cells mitosis is initiated by the activation of the cdc2 protein kinase, the catalytic subunit of mitosis promoting factor (MPF) [reviewed in Nurse, 1990]. The activity of the cdc2 protein kinase is periodically regulated throughout the cell cycle both by its association with regulatory subunits (cyclins) (Hunt, 1991) and by phosphorylation on threonine, tyrosine, and serine residues (Draetta and Beach, 1988; Draetta et al., 1988; Gautier et al., 1989; Morla et al., 1989; Gould and Nurse, 1989). The major kinase involved in the transition from G_2 to M phase is a complex between cdc2 and cyclin B (Nurse, 1990). During the transition from S to G_2 to prophase, cyclin B accumulates and associates with cdc2. The kinase undergoes phosphorylation at Thr-161 (Ducommun et al., 1991; Gould et al., 1991; Krek and Nigg, 1991), which stabilizes cyclin binding and is required for subsequent kinase activity. Additional phosphorylation occurs at residues Thr-14 and Tyr-15, which are located in the N-terminal region of the cdc2 molecule close to the ATP-binding site. This modification prevents immediate activation of the kinase, which is stored as an inactive *pre-MPF* or *prokinase*. The phosphorylation state of these residues is important in coupling DNA replication and mitosis. Studies by Dasso and Newport (1990) using *Xenopus* egg extracts that cycle between S and M phase suggest that tyrosine dephosphorylation of cdc2 is sensitive to completion of S phase. In fission yeast, tyrosine phosphorylation on cdc2 prevents the premature occurrence of mitosis before DNA replication has been completed. The substitution of the Tyr-15 residue with phenylalanine advances cells prematurely into mitosis and renders them unable to arrest in response to drugs that inhibit DNA synthesis (Gould et al., 1990). Two functionally overlapping kinases known as wee1 and mik1 in fission yeast mediate the suppression of cdc2 by tyrosine phosphorylation (Russell and Nurse, 1987; Lundgren et al., 1991). The human wee1 kinase has been shown

to phosphorylate cdc2 on Tyr-15 *in vitro* and to inactivate the kinase (Parker and Piwnica-Worms, 1992; McGowan, 1993). The phosphorylation on Thr-14 is believed to be catalyzed by a distinct kinase (Kornbluth, 1994). Conversely, the cdc25 phosphatase relieves this inhibition by serving as a cdc2-specific phosphatase, activating the kinase by removing the phosphate residues on Tyr-15 and possibly Thr-14 (Dunphy and Kumagai, 1991; Gautier et al., 1991; Lee et al., 1992; Millar et al., 1991a; Strausfeld et al., 1991).

The first evidence for a role of cdc25 in mitosis was provided by (Fantes, 1979) who showed that the G_2 cell cycle arrest caused by *cdc25* mutations in *Schizosaccharomyces pombe* is suppressed by inactivation of *wee1*. This observation was explained in later experiments showing that the cdc25 protein is a dose-dependent inducer of mitosis that functions in opposition to *wee1* and *mik1* (Russell and Nurse, 1986; Lundgren et al., 1991). Overexpression of cdc25 in fission yeast correlates with a decreased level of tyrosine phosphorylation of cdc2. This results in a premature entry into M phase and abolishes the dependence of M phase on completion of S phase (Gould et al., 1990; Enoch and Nurse, 1990). Recently, a second protein tyrosine phosphatase (PTPase) has been described in fission yeast, encoded by the *pyp3* gene that also contributes to tyrosyl dephoshosphorylation and activation of cdc2 (Millar et al., 1992). This PTPase acts cooperatively with cdc25 in *S. pombe*. Surprisingly, pyp3 bears little similarity to the cdc25 class of phosphatases and is most similar to protein tyrosine phosphatase 1B (PTP1B) (Tonks et al., 1988).

It seems that the transition from G_2 to M phase is gated by a molecular switch involving a change in the relative kinase and phosphatase activities that regulate the phosphorylation of cdc2 on Thr-14 and Tyr-15. The subject of this article is the recent advances in understanding the onset of mitosis in higher eukaryotes mediated by a regulation of the phosphatase activity of the mitotic inducer cdc25.

II. ISOLATION OF *CDC25* GENES AND EXPRESSION PATTERN OF THEIR GENE PRODUCTS

The first *cdc25* gene, *cdc25*$^+$, was cloned from *S. pombe* by complementation of a temperature-sensitive mutant strain *cdc25-22* (Russell and Nurse, 1986).

```
(Dmcdc25)   E T V A R L L K G E F S D K V A S - - - Y R I I D C R Y P Y E F E G G H I E G A K N L Y T T E Q I L   349
(Dmtwine)   D T L A R L I Q G E F D E Q L G S Q G G Y E I I D C R Y P Y E F L G G H I R G A K N L Y T R G Q I Q   298
(Xlcdc25a)  E T L A A L I H G D F S S L V E K - - - I F I I D C R Y P Y E Y D G G H I K G A L N L H R Q E E V T   434
(Xlcdc25b)  E T L A A L M H G D F N S L V E K - - - F F I I D C R Y P Y E Y D G G H I K S A F N L H R Q E E V T   483
(Xlcdc25c)  E T L A A L M H G D F N S L V E K - - - F F I I D C R Y P Y E Y D G G H I K S A F N L H R Q D E V T   453
(Hscdc25c)  E T V A A L L S G K F Q G L I E K - - - F Y V I D C R Y P Y E Y L G G H I Q G A L N L Y S Q E E L F   354
(Hscdc25b)  E T M V A L L T G K F S N I V D K - - - F V I V D C R Y P Y E P E G G H I K T A V N L P L E R D A E   450
(Mmcdc25b)  E T M V A L L T G K F S N I V E K - - - F V I V D C R Y P Y E Y E G G H I K N A V N L P L E R D A E   460
(Hscdc25a)  E I M A S V L N G K F A N L I K E - - - F V I I D C R Y P Y E Y E G G H I K G A V N L H M E E E V E   408
(Spcdc25)   E T L L G L L D G K F K D I F D K - - - C I I I D C R F E Y E Y L G G H I S T A V N L N T K Q A I V   462
(Scmih1)    N N M C E S F Y N S - - - - - - - - - - C R I I D C R F E Y E Y T G G H I I N S V N I H S R D E L E   294

(Dmcdc25)   D E F L T V Q Q T E L Q Q Q Q N A E S G H K R N I I I F H C E F S S E R G P K M S R F L R N L D R E   399
(Dmtwine)   E A F P T L - - - - - - - - - - T S N Q E N R R I Y V F H C E F S S E R G P K L L R Y L R S N D R S   338
(Xlcdc25a)  D Y F - - - - - - - L K Q P L T P T M A Q K R L I I I F H C E F S S E R G P K M C R F L R E E D R A   477
(Xlcdc25b)  D Y F - - - - - - - L Q Q P L T P L M V Q K R L I I I F H C E F S S E R G P K M C R F L R E E D R A   526
(Xlcdc25c)  D Y F - - - - - - - L Q Q P L T P L M A Q K R L I I I F H C E F S S E R G P K M C R S L R E E D R A   496
(Hscdc25c)  N F F - - - - - - - L K K P I V P L D T Q K R I I I V F H C E F S S E R G P R M C R C L R E E D R S   397
(Hscdc25b)  S F L - - - - - - - L K S P I A P C S L D K R V I L I F H C I F S S E R G P R M C R F I R E R D R A   493
(Mmcdc25b)  T F L - - - - - - - L Q R P I M P C S L D K R I I L I F H C E F S S E R G P R M C R F I R E R D R A   503
(Hscdc25a)  D F L - - - - - - - L K K P I V P T D - G K R V I V V F H C E F S S E R G P R M C R Y V R E R D R L   450
(Spcdc25)   D A F - - - - - - - L S K P L T - - - - - H R V A L V F H C E H S A H R A P H L A L H F R N T D R R   500
(Scmih1)    Y E F I - - - - H K V L H S D T S N N N T L P T L L I I H C E F S S H R G P S L A S H L R N C D R I   340
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{Dmcdc25}   R N T N A Y P A L H Y P E I Y L L H N G Y K E F F E S H V E L C E P H A Y R T M L D - - - - - - - -   441
{Dmtwine}   Q H T H N Y P A L D Y P E L Y I L H N G Y K E F F G L Y S Q L C Q P S Q Y V P M L A - - - - - - - -   380
{Xlcdc25a}  - - R N E Y P S L Y Y P E L Y L L K G G Y K D F F P E Y K E L C E P Q S Y C P M H H - - - - - - - -   517
{Xlcdc25b}  - - S N D Y P S L Y Y P E L Y L L K G G Y K D F F P E Y K E L C E P Q S Y C P M H H - - - - - - - -   566
{Xlcdc25c}  - - S N D Y P S L Y Y P E L Y L L K G G Y K D F F P E Y K E L C E P Q S Y C P M H H - - - - - - - -   536
{Hscdc25c}  - - L N Q Y P A L Y Y P E L Y I L K G G Y R D F F P E Y M E L C E P Q S Y C P M H H - - - - - - - -   437
{Hscdc25b}  - - V N D Y P S L Y Y P E M Y E L K G G Y K E F F P Q H P N F C E P Q D Y R P M N H - - - - - - - -   533
{Mmcdc25b}  - - A N D Y P S L Y Y P E M Y I L K G G Y K E F F P Q H P N F C E P Q D Y R P M N H - - - - - - - -   543
{Hscdc25a}  - - G N E Y P K L H Y P E L Y V L K G G Y K E F F M K C Q S Y C E P P S Y R P M H H - - - - - - - -   490
{Spcdc25}   M N S H R Y P F L Y Y P E V Y I L H G G Y K S F Y E N H K N R C D P I N Y V P M N D R S H V M T C T   550
{Scmihl}    I N Q D H Y P K L F Y P D I L I L D G G Y K A V L T - F P E L C Y P R Q Y V G M N S Q E N L L N C E   389
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Figure 1. Alignment of the catalytic domain of cdc25^{+} protein homologs from *Drosophila string* (Dmcdc25), *Drosophila twine* (Dmtwine), human (Hscdc25a, hscdc25b, hscdc25c), mouse (Mmcdc25b), *Xenopus* oocytes (Xlcdc25a, Xlcdc25b, Xlcdc25c), *S. pombe* (Spcdc25), and *S. cerevisiae* (Scmih1) using the FASTA program. Identical amino acids are boxed. Dashes within the sequences indicated individual amino acid gaps created by the computer to generate optimal alignment.

Another *cdc25* homologue from the highly divergent budding yeast *Saccharomyces cerevisiae* was cloned by rescue of a fission yeast temperature sensitive (ts) *cdc25ts* mutation and called *MIH1* (Russell et al., 1989). The fact that the mitotic control elements also exist in *S. cerevisiae* made it likely that similar control elements also exist in higher eukaryotes. Russell and coworkers cloned the first human *cdc25* gene, which is called cdc25-C according to the present nomenclature (Sadhu et al., 1990). Additional human *cdc25* genes, named *cdc25-A* and *cdc25-B*, were cloned by Galaktionov and Beach (1991) and Nagata et al. (1991). Sequence comparisons of the C terminus of the cdc25-C and cdc25-A proteins reveal 48% similarity, while cdc25-B and cdc25-C share 43% similarity. The mRNA expression of cdc25-B (cdc25Hu2) in human cell lines is 10 to 100 times higher than cdc25-C (cdc25Hu1). It has been found also to be particularly high in some cancer cells, including SV40 transformed fibroblasts (Nagata et al., 1991). Recently a structural and functional homologue of the human cdc25-B phosphatase has been cloned from a mouse carcinoma cell line (cdc25M2) (Kakizuka et al., 1992). This mouse homologue is expressed in a developmental and cell cycle dependent fashion, suggesting that the expression and accumulation of the cdc25 mitotic inducer may play a critical role in the regulation of mouse development.

Three different cdc25 genes have been described in *Xenopus* (Kumagai and Dunphy, 1992; Izumi et al., 1992). They are all highly homologous to the human cdc25-C protein. Two *cdc25* genes have been isolated in *Drosophila*. The first cdc25 protein is the product of the *string* (stg) gene (Edgar and O'Farrell, 1989). Embryos lacking string function proceed through the first 13 rapid mitotic cycles of the syncytial embryo utilizing maternally provided gene products and then arrest in the G_2 stage of cycle 14, the first division after cellularization. The second homologue is the product of the *twine* gene (Alphey et al., 1992) and has a function in the male and female germline of *Drosophila*. *Twine* transcripts have been detected in the growing stage of premeiotic cysts. Analysis of a *twine* mutant suggests a requirement for the gene in oogenesis. All the *cdc25* genes named here can rescue a temperature-sensitive *S. pombe* mutation. However, higher eukaryotes seem to have a greater complexity of cdc25 molecules than *S. pombe*, which might have different functions and may be regulated in different ways. Figure 1 shows a sequence comparison of the catalytic domain among the cdc25 homologues.

III. CDC25 PHOSPHATASE: SUBSTRATES AND SPECIFICITY

The C-terminal catalytic domains of cdc25 molecules contain conserved amino acids and share a small degree of homology to the conserved active site of protein tyrosine phosphatases (IVXHCXXXXXR) (Strausfeld et al., 1991). This cysteinyl residue in PTPases is believed to be required for the formation of an unstable thiophosphate bond with the substrate. When the corresponding residue in cdc25 (Cys-480 in *Drosophila string*) is mutated to serine or alanine the phosphatase activity of cdc25 is completely abolished (Gautier et al., 1991), suggesting that it has a similar catalytic mechanism to other protein tyrosine phosphatases. The N-terminal halves of cdc25 homologues from different species share little sequence similarity. However, several short stretches of amino acids are conserved. For example, putative nuclear targeting sequences are found in *Xenopus cdc25* clones as well as those from other species (Izumi et al., 1992). Several putative phosphorylation sites for cdc2 kinase are also found in the vicinity of this motif. Substitution of another conserved residue in the putative catalytic site of cdc25, Arg-385, with Lys or Met, also resulted in an inactive protein product. His-479, located close to the cysteine residue, is not essential for mammalian PTPase function, and mutation of this residue to alanine resulted in a cdc25 protein that is still able to dephosphorylate cdc2. The residues between the catalytic cysteine and arginine, that differ between cdc25s and other PTPases, form a loop that may be involved in the differential recognition of the phosphorylated Tyr-15 residue in cdc2 (Gautier et al., 1991).

The cdc2 kinase in higher eukaryotic cells is kept in an inactive state by phosphorylation on both Tyr-15 and Thr-14 residues. Dephosphorylation of Tyr-15 alone is not sufficient to activate the kinase. The discovery that the phosphatase VH1 from vaccinia virus (the closest relative to the cdc25 class) displays catalytic activity toward both phosphoserine and phosphotyrosine (Guan et al., 1991), raises the question of whether cdc25 can dephosphorylate cdc2 on both tyrosine and threonine residues. The activation of vertebrate cdc2 has been shown to be promoted by cdc25, which is phosphorylated on Tyr-15 and Thr-14 (Gautier et al., 1991; Kumagai and Dunphy, 1991; Strausfeld et al., 1991). This suggests that cdc25 can dephosphorylate cdc2 on Thr-14 as well. Dual-specific activity against both tyrosine and serine/threonine residues is an unusual feature of the cdc25 phosphatase in higher eu-

karyotes, since there has been only a classification of either tyrosine phosphatases or serine/threonine phosphatases.

A striking characteristic of cdc25 is its high specificity towards its substrate cdc2. Other substrates including the phosphorylated 11-mer peptide containing Tyr-15, fragments of cdc2, or known general phosphatase substrates like *para*-nitrophenylphosphate (pNPP) are poor substrates of full-length cdc25 (Gautier et al., 1991). The high-substrate specificity of cdc25 is unusual among phosphatases. For instance, the T cell PTPase acts on a variety of substrates and can replace cdc25 in yeast (Tonks et al., 1988; Gould et al., 1990). The substrate specificity of cdc25 might be regulated by the N-terminal part of the phosphatase. The first attempts using full-length *Drosophila* or human cdc25 proteins failed to detect any activity using pNPP, despite the fact that these enzymes were quite effective in dephosphorylating and activating the cdc2 kinase (Kumagai and Dunphy, 1991; Strausfeld et al., 1991). One possible explanation is that the N-terminal regulatory domain of cdc25 negatively regulates the catalytic domain.

Another member of the cyclin-dependent kinase family, cdk2, is also dephosphorylated and activated by cdc25-C *in vitro* (Gabrielli et al., 1992).

IV. CELL-CYCLE REGULATION OF *CDC25* PHOSPHATASE

The phosphorylation state of the inhibitory sites in the cdc2 kinase is responsive to events such as DNA replication, providing a mechanism by which activation of the cdc2/cyclin B kinase and entry into mitosis do not occur prematurely before other essential cell cycle events have been completed. This regulation may occur through changes in activity of the opposing kinases and phosphatases that act on the phosphorylation sites. In fission yeast, the cdc25 protein has been found to be cell cycle regulated, oscillating in abundance during the cell cycle and reaching a peak at the G_2/M transition (Ducommun et al., 1990; Moreno et al., 1990). The mRNA levels of cdc25 fluctuate with a peak at mitosis. In HeLa cells cdc25-C mRNA levels are very low in G_1 and increase approximately fourfold as cells progress towards M phase (Sadhu et al., 1990). However, the protein levels remain constant during the cell cycle, fluctuating less than twofold. The first evidence that cdc25 might be regulated by phosphorylation came from studies in fission yeast, showing that cdc25 is a phosphoprotein (Ducommun et al., 1990; Moreno et al., 1990). More recent evidence has come from experiments in *Xenopus*

eggs and human (Izumi et al., 1992; Kumagai and Dunphy, 1992; Hoffmann et al., 1993). Both in human and in *Xenopus* egg extracts, cdc25 of the C-type has a detectable phosphatase activity in interphase, but does not induce tyrosine dephosphorylation on cdc2. One possible explanation for this is that the opposing Tyr kinase may overwhelm the low activity of the interphase cdc25. The activity of cdc25-C increases at the onset of mitosis both in cycling *Xenopus* egg extracts and HeLa cell extracts. This elevation in phosphatase activity is mediated by phosphorylation on serine and threonine residues (Kumagai and Dunphy, 1992). When cdc25-C is phosphorylated in mitotic HeLa cell extract or by purified cdc2/cyclin-B kinase, its catalytic activity is increased up to 15-fold. Cyclin A-dependent kinases (cdc2 and cdk2) did not phosphorylate cdc25-C (Hoffmann et al., 1993). Human cdc25-C can activate cdc2/cyclin B1 in *Xenopus* egg extracts and induce *Xenopus* oocycte

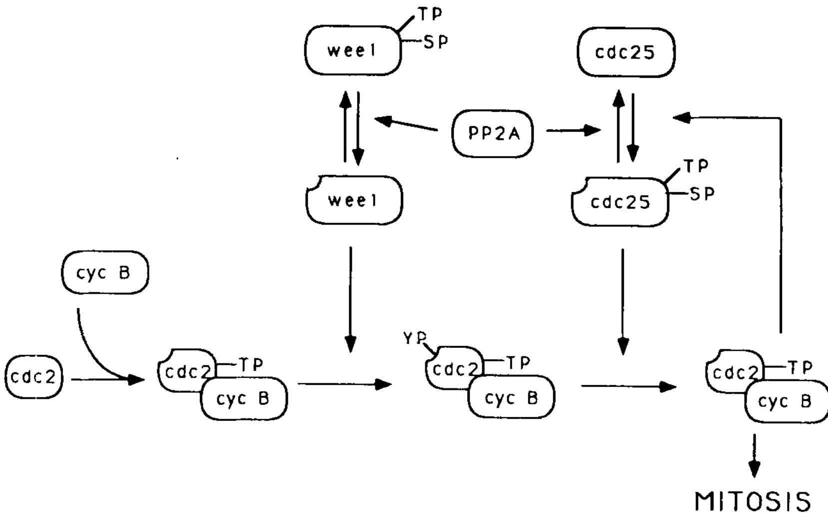

Figure 2. Model for the positive feed-back activation of cdc2/cyclin B at the transition from G_2 to M phase. The cdc2/cyclin B kinase is activated following dephosphorylation of Tyr-15 (and Thr-14). Generation of small amounts of active cdc2/cyclin B phosphorylates the cdc25 phosphatase directly. Thus, a positive feedback loop is created leading to the entry into mitosis. In addition a type-2A phosphatase activity towards cdc25-C is specifically inhibited by phosphorylation. The dephosphorylation of wee1 by a type 2A-phosphatase is not demonstrated and remains speculative.

maturation; this is possible only after stable thiophosphorylation. Thus it seems that phosphorylation of cdc25-C by cdc2/cyclin B forms part of an autoamplification loop during the process of MPF activation observed *in vivo* (Figure 2). Furthermore, *Xenopus* cdc25 activity is negatively regulated by an okadaic acid and microcystin-sensitive phosphatase, which functions to maintain cdc25 in its weakly active 70 kDa form (Kumagai and Dunphy, 1992; Izumi et al., 1992; Hoffmann et al., 1993). Both phosphatase-1 (PP-1) and phosphatase-2A (PP-2A) can remove phosphate from mitotically activated cdc25 protein and inactivate it *in vitro* (Izumi et al., 1992). However, in *Xenopus* interphase extracts inhibition of PP-2A by okadaic acid completely blocks cdc25-C dephosphorylation, whereas inhibition of PP-1 by using the PP-1 specific inhibitor-2 has no effect (Clarke et al., 1993). Therefore, the phosphatase that dephosphorylates cdc25-C in egg extracts is PP-2A-like.

Recently Seki et al. (1992) cloned the cdc25-C protein from hamster and found that it is required for an activation of cdc2 kinase caused by loss of RCC1 (regulator of chromosome condensation function), resulting in a premature chromosome condensation (PCC). An apparent increase in molecular mass of the cdc25-C protein due to phosphorylation is found in cells that show premature chromosome condensation and depends on activation of cdc2 kinase. Cdc25-C seems to be synthesized upon loss of RCC1 function.

V. SUBCELLULAR LOCALIZATION OF CDC25 PROTEINS

Studies on the subcellular localization of the human cdc25 protein should discern where activation of the cdc2/cyclin B complex occurs. Immunofluorescence studies using anti-cdc25-C peptide antibodies in HeLa cells showed a clear nuclear staining of the protein (Millar et al., 1991b). In cells undergoing mitosis, strong staining occurred throughout most areas of the cell but was excluded from the mitotic chromosome. Only a weak cytoplasmic staining was observed. Additional studies were carried out by indirect immunofluorescence with a synthetic peptide against the phosphatase consensus site of cdc25, which probably cross-reacts with at least two different human cdc25 homologues (Girard et al., 1992). Cdc25 proteins were found to be essentially localized in the nucleus throughout interphase and early prophase. Just before the completion of nuclear envelope breakdown at the prophase–prometaphase boundary, cdc25 proteins were redistributed throughout the cytoplasm.

The enzyme–substrate relationship between cdc25 and cdc2 predicts that the two proteins might interact at least transiently. This interaction was demonstrated in *Xenopus* oocyte extracts and is about fourfold higher in mitotic than in interphase extracts (Gautier et al., 1991; Jessus and Beach, 1992). This association might be weak or transient, because only a fraction of the cdc25 protein is present in the complex (Gautier et al., 1991).

The cdc25 phosphatase must presumably interact with cdc2/cyclin B at the onset of mitosis. Cyclin B1, however, has been found to be cytoplasmic throughout S and G_2 phases (Gautier and Maller, 1991; Pines and Hunter, 1991). During the entry into mitosis, when chromatin has started to condense but before nuclear envelope breakdown, a large proportion of cyclin B enters the nucleus (Pines and Hunter, 1991). In *Xenopus* cell-free extracts cdc2 is not dephosphorylated on tyrosine if nuclear transport is inhibited with wheat germ agglutinin (Kumagai and Dunphy, 1991). Because the cdc25-C phosphatase is primarily nuclear, it is possible that cdc2 is dephosphorylated by cdc25 after transport to the nucleus. It has yet to be confirmed that a colocalization of cyclin B and cdc25-C in the nucleus exists, and it has already been shown that constitutive translocation of a cyclin B due to an heterologous nuclear translocation signal does not cause premature entry into mitosis (E. Nigg, personal communication). Alternatively, activation of cdc2/cyclin B in the cytoplasm may be triggered by transport of cdc25-C out of the nucleus.

VI. CONCLUSIONS

The finding that the phosphatase activity of cdc25 is cell cycle regulated has improved our understanding of the molecular events that are responsible for the onset of mitosis. The involvement of cdc2 in the stimulation of cdc25 provides a simple mechanism for the autocatalytic amplification of MPF activity. However, it cannot be excluded that another kinase(s) present in mitotic extracts also contributes to the activation of cdc25. It remains unclear how the initiation of the autocatalytic amplification of cdc2/cyclin B is mediated.

The existence of multiple cdc25 genes in higher eukaryotes has been reported (Galactionov and Beach, 1991; Kumagai and Dunphy, 1992; Izumi et al., 1992; Nagata et al., 1991). It remains to be elucidated whether their gene products have different functions, for example, in their substrate specificities or subcellular localizations.

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CONTROL OF NUCLEAR LAMINA ASSEMBLY/DISASSEMBLY BY PHOSPHORYLATION

Matthias Peter and Erich A. Nigg*

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ABSTRACT

The nuclear envelope is a complex organelle that separates the nuclear and cytoplasmic compartments and plays a key role in communication between them. It consists of a double nuclear membrane, nuclear pore complexes, and the nuclear lamina.

During mitosis, the nuclear envelope transiently disassembles, and recent results suggest that site specific phosphorylation of the nuclear lamins controls the polymerization state of the lamina during the cell cycle. The focus of this review is to summarize the different cell cycle dependent phosphorylations of the nuclear lamin proteins. In particular, we will emphasize the evidence leading to the conclusion that the mitotic lamina dissassembly might be triggered by direct phosphorylation of the nuclear lamins by cdc2 kinase, a major regulator of the eukaryotic cell-cycle (for review see Draetta 1990; Hunt 1991; Norbury and Nurse 1992; Kirschner 1992).

I. INTRODUCTION

The nuclear lamina is a protein meshwork underlining the inner nuclear membrane. Its major constituents, the nuclear lamins, share structural homologies with a class of proteins called the intermediate filaments (McKeon et al., 1986; Fisher et al., 1986; Aebi et al., 1986; for review see Parry et al., 1986; Franke, 1987). Lamins consist of a central α-helical rod domain that is flanked by non-α-helical domains at the amino- and carboxy-terminal ends (Figure 1, for review see Gerace and Burke, 1988; Nigg, 1989; McKeon, 1991). In contrast to cytoplasmic intermediate filaments, nuclear lamins contain a prototypic SV40 large T antigen-type nuclear location signal (Loewinger and McKeon, 1988) and a CaaX-box at the carboxy terminus (Figure 1). The latter motif, which is also found in yeast a-mating pheromone, *ras* proteins, and many other GTP-hydrolyzing proteins (for review see Glomset et al. 1990; Maltese 1990) is subject to a complex series of posttranslational modifications, including isoprenylation, proteolytic trimming, and carboxyl methylation (Chelsky et al., 1987, 1989; Beck et al., 1988, 1990; Wolda and Glomset, 1988; Farnsworth et al., 1989; Vorburger et al., 1989; Kitten and Nigg, 1991). These processing events are important for targeting newly synthesized lamins to the nuclear envelope (Holtz et al., 1989; Krohne et al., 1989; Kitten and Nigg, 1991).

Based on biochemical properties and structural criteria, most lamins can be classified as either A-type or B-type (Gerace and Blobel, 1980;

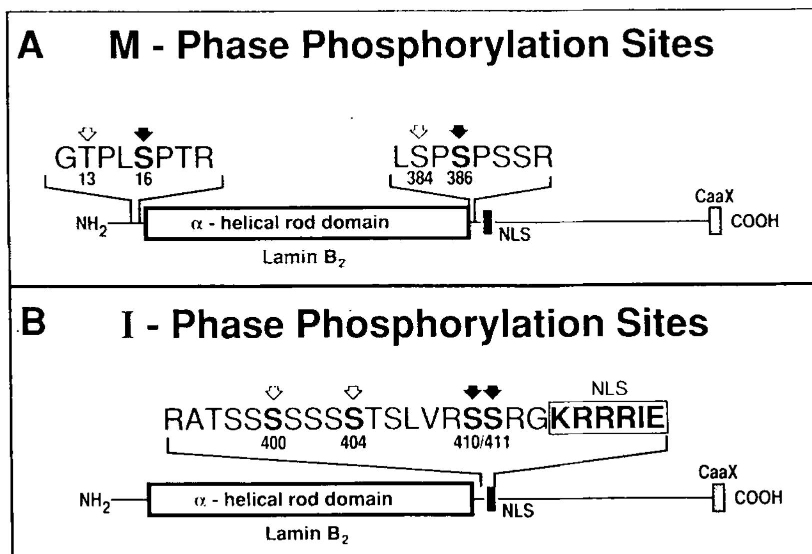

Figure 1. Phosphorylation of chicken lamin B_2 during interphase and mitosis. Indicated in these schemes are residues known to be phosphorylated during mitosis (Panel A) or interphase (Panel B). For detailed discussion see Peter et al., 1990 and Hennekes et al., 1993. Major phosphorylation sites are indicated by filled arrows, minor sites by open arrows. NLS: Nuclear Localization Signal; CaaX (C = cysteine, A = aliphatic, X = any residue): C-terminal motif, undergoing farnesylation and carboxymethylation. Note that additional phosphorylation sites have been described in human and murine lamin C (Ward and Kirschner, 1990; Eggert et al., 1991).

Burke and Gerace, 1986; Krohne et al., 1987; Höger et al., 1988, 1990; Peter et al., 1989; for review see Gerace and Burke, 1988; Nigg, 1989). Expression of at least two B-type lamins is characteristic of most vertebrate cell types, whereas little or no A-type lamins can be detected in early embryos or undifferentiated pluripotent cell lines. Disassembled B-type lamins remain associated with the nuclear membrane throughout the cell cycle, but A-type lamins are completely solubilized during mitosis.

During interphase the nuclear lamina is believed to be important for nuclear envelope integrity and the organization of chromatin (for review see Gerace and Burke, 1988; Nigg, 1989; McKeon, 1991). In support of this notion, purified lamins are able to bind to isolated metaphase

chromosomes (Burke, 1990; Glass and Gerace, 1990) or chromatin preparations *in vitro* (Yuan et al., 1991; Höger et al., 1991). Recently, it has been demonstrated that lamin B_1 directly interacts with chromatin scaffold attachment regions, suggesting that the lamina might anchor chromatin at the nuclear envelope (Ludérus et al., 1992). Evidence is also emerging that an intact nuclear lamina is important to allow DNA replication (Blow and Laskey, 1988; Meier et al., 1991; Leno and Laskey, 1991, for review see Leno, 1992).

II. CELL CYCLE DEPENDENT PHOSPHORYLATION OF THE NUCLEAR LAMINS

A. Phosphorylation of the Nuclear Lamins During Mitosis

During mitosis, cell nuclei undergo dramatic changes in their structural organization: DNA condenses to form metaphase chromosomes, the microtubule network rearranges and forms a mitotic spindle, and in many cells the nuclear envelope including the nuclear lamina disassembles. Nuclear envelope disassembly has been a key indicator event to monitor M phase. It is important to distinguish lamina disassembly and nuclear envelope breakdown. Nuclear envelope breakdown includes depolymerization of the lamina, disassembly of the nuclear membrane and the nuclear pore complexes. It is clear that depolymerization of the nuclear lamina alone is not sufficient to cause nuclear envelope breakdown, but is a necessary step (Peter et al., 1990; Heald and McKeon, 1990; Lamb et al., 1990; Pfaller et al., 1991; for review see Nigg, 1992).

Hyperphosphorylation of the nuclear lamins has long been suspected to trigger the transient breakdown of the nuclear lamina (for review see Gerace and Burke, 1988), and recent evidence supports this notion. Mitosis-specific phosphorylation sites have been identified on both human lamin C (Ward and Kirschner, 1990) and chicken lamin B_2 (Peter et al., 1990). The major phosphorylated residues are located on both sides of the α-helical central rod domain (Figure 1, panel A), which is involved in coiled–coil interactions between lamin molecules. These sites are conserved in all the nuclear lamins that have been sequenced (Heald and McKeon, 1990; Nigg et al., 1991) but are absent from cytoplasmic intermediate filaments. Mutational analysis revealed that a subset of these M-phase-specific phosphorylation sites are critical for lamina disassembly both *in vivo* (Heald and McKeon, 1990) and *in vitro* (Peter et al., 1991).

B. Phosphorylation of Lamins During Interphase

Besides phosphorylation of the mitotic phosphorylation sites, lamin molecules are phosphorylated throughout the cell cycle (Ottaviano and Gerace, 1988; Hornbeck et al., 1988; Peter et al., 1990; Nigg et al., 1991). However, the function(s) of these interphase phosphorylations is not understood. Interestingly, not only are new sites phosphorylated at the onset of mitosis (see above), but prominent interphase phosphorylation sites are *de*phosphorylated (Peter et al., 1990). These latter sites can readily be phosphorylated *in vitro* by purified protein kinase C or by purified protein kinase A (Peter et al., 1990; Eggert et al., 1991; Hennekes et al., 1993). Neither of these two kinases is able to disassemble embryonic nuclei or lamin head-to-tail polymers (Peter et al., 1990; Hennekes et al., 1993), and it is clear that the sites phosphorylated by protein kinase C and protein kinase A differ from the mitotic phosphorylation sites involved in disassembly of the nuclear lamina (Peter et al., 1990; Henneckes et al., 1993). However, recent evidence suggests, that in addition to phosphorylation of mitotic specific sites, *de*phosphorylation of these interphase phosphorylation sites may also play an important role for nuclear lamina breakdown (Lamb et al., 1991; Molloy and Little, 1992). One of these sites has been identified on chicken lamin B_2 in a region after the central rod domain of the lamin molecule (Figure 1, panel B; Henneckes et al., 1993).

B-type lamins are also phosphorylated in response to tumor promotors such as the phorbol ester PMA (Hornbeck et al., 1988; Fields et al., 1988, 1991; Peter et al., 1990; Tsuda and Alexander, 1990; Kasahara et al., 1991; Heneckes et al., 1993). Protein kinase C is activated in response to PMA and is able to phosphorylate a site on B-type lamins that is specifically phosphorylated after exposure of cells to PMA *in vivo* (Hornbeck et al., 1988; Peter et al., 1990; Henneckes et al., 1993). Interestingly, this PMA-induced phosphorylation site is located close to the nuclear localization signal, suggesting that phosphorylation of this site might influence the nuclear uptake of newly synthesized lamin molecules (Hennekes et al., 1993, see also Rihs and Peters, 1989; Moll et al., 1991). Recent results also indicate that phosphorylation of lamin B by protein kinase A and protein kinase C might regulate the interaction with plectin (Foisner et al., 1991). A summary of the different lamin phosphorylation sites during the cell cycle is presented in Figure 1.

III. *CDC2* KINASE DIRECTLY TRIGGERS LAMINA DISASSEMBLY DURING MITOSIS

Available evidence indicates that cdc2 kinase, a major regulator of the eukaryotic cell cycle, controls mitotic lamina disassembly by acting directly on lamin proteins. Highly purified cdc2 kinase ($p34^{cdc2}$-cyclin B complex) is able to phosphorylate lamins on M-phase-specific sites and concomitantly able to trigger lamina disassembly when incubated with isolated embryonic nuclei (Peter et al., 1990; Dessev et al., 1991). Furthermore, cell-free extracts depleted of cdc2 kinase are no longer able to disassemble the nuclear envelope of exogenously added nuclei *in vitro* (Dessev et al., 1991). In addition, purified cdc2 kinase is able to disassemble lamin head-to-tail polymers reconstituted from bacterially expressed lamin proteins (Heitlinger et al., 1991; Peter et al., 1991). Importantly, mutations of the major phosphorylation sites of cdc2 kinase confer resistance to lamina disassembly both *in vivo* (Heald and McKeon, 1990) and *in vitro* (Peter et al., 1991).

Genetic support for a direct role of cdc2 kinase in phosphorylation of the nuclear lamina was obtained from a fission yeast strain expressing chicken lamin B_2 (Enoch et al., 1991). The expressed chicken lamin B_2 assembled in a ringlike structure around the yeast nucleus with biochemical properties similar to the lamina of higher eukaryotes. Moreover, this laminalike structure was shown to disassemble during mitosis. Interestingly, chicken lamin B_2 was phosphorylated in a cell cycle dependent manner in this fission yeast strain, and phosphorylation was temperature sensitive in a strain bearing a temperature-sensitive mutation in the cdc2 gene (Enoch et al., 1991). The most straightforward interpretation of these results is that cdc2 kinase acts directly on the nuclear lamins.

Similar to the role of cdc2 kinase in triggering mitotic lamina disassembly, evidence is implicating cdc2 kinase in directly causing disassembly of cytoplasmic intermediate filaments during mitosis (Chou et al., 1990; for review see Erikson et al., 1992). A mitosis specific phosphorylation site has been identified in the amino terminus of vimentin, and the same site is phosphorylated *in vitro* by purified cdc2 kinase (Chou et al., 1991). Therefore, it appears that cells use similar mechanisms to regulate the assembly state of nuclear lamins and at least some of the cytoplasmic intermediate filaments during mitosis.

Nevertheless, it would be premature to exclude the possibility that other kinases may contribute to induce lamina dissassembly *in vivo*. For instance, human lamin C is also phosphorylated on Ser-404 during

mitosis *in vivo*, and this site can be phosphorylated *in vitro* by purified S6 kinase, but not by cdc2 kinase (Ward and Kirschner, 1990). S6 kinase is activated during mitosis in a cdc2-dependent manner (Erikson and Maller, 1991; Ferell et al., 1991). Thus, cdc2 kinase may act on nuclear lamins both directly and indirectly by activation of downstream kinases (for review see Nigg, 1991). However, to date no specific role of this S6 phosphorylation site in lamina disassembly has been reported, and, at least *in vitro*, phosphorylation of the nuclear lamins by cdc2 kinase is sufficient to trigger disassembly (Peter et al., 1990, 1991; Dessev et al., 1991).

Recent results also indicate that MAP kinases (mitogen activated kinases, for review see Blenis, 1991; Crews et al., 1992; Pelech and Sanghera, 1992) are capable of phosphorylating chicken lamin B_2 specifically on the N-terminal mitotic phosphorylation site (Peter et al., 1992). In addition, MAP kinases are able to disassemble preformed lamin head-to-tail polymers *in vitro*. Thus, members of the MAP kinase family display a substrate specificity overlapping that of cdc2 kinase (see also Lewis-Clark et al., 1991; Shangera et al., 1990). MAP kinases have been shown to be activated during meiotic cell divisions (Gotoh et al., 1991a,b; Posada et al., 1991; Ferell et al., 1991), and it will be important to determine whether MAP kinases are also activated during mitosis. It is clear however that MAP kinases are activated in response to a variety of mitogens and it is conceivable, therefore, that MAP kinases might be involved in regulating the plasticity of the nuclear lamina by partially and/or locally depolymerizing the nuclear lamina during interphase.

IV. STRUCTURAL CONSEQUENCES OF MITOTIC LAMINA PHOSPHORYLATION BY *CDC2* KINASE

Recent experiments have demonstrated that the nuclear lamina is assembled through three distinct levels of structural organization that appear to be maintained by interactions involving different domains of the lamin molecules (Heitlinger et al., 1991; Gieffers and Krohne, 1991; Peter et al., 1991, for review see Dessev, 1992). At the first level of structural organization, two lamin molecules assemble in a parallel, unstaggered orientation to form lamin dimers. This interaction is mediated by the central α-helical domain of the lamin molecule. At the second level of organization, lamin dimers associate longitudinally to form head-to-tail polymers (Figure 2). This association involves the amino-terminal head domain, since truncated lamins lacking the amino terminus are unable to

associate longitudinally but are still capable of forming lamin dimers (Heitlinger et al., 1992). In contrast, lamin molecules lacking the carboxy-terminal domain still assemble into head-to-tail polymers (Gieffers and Krohne, 1991; Heitlinger et al., 1992). Finally, such head-to-tail polymers further associate laterally to form thicker filamentous structures in which the lamin dimers are arranged in a half-staggered manner (Heitlinger et al., 1991, 1992).

Phosphorylation of such preformed head-to-tail lamin polymers by cdc2 kinase results in disassembly of the polymers into lamin dimers (Peter et al., 1991). Similarly, lamin dimers phosphorylated by cdc2 kinase fail to assemble into head-to-tail polymers (Heitlinger et al., 1991). These results suggest that conformational changes induced by phosphorylation of the amino-terminal cdc2 phosphorylation site interfere with the longitudinal association of lamin dimers into head-to-tail polymers but have little effect on the α-helical part of the lamin dimer (Figure 2). Lamin dimers are also predominantly found during mitosis *in vivo* (Dessev et al., 1990).

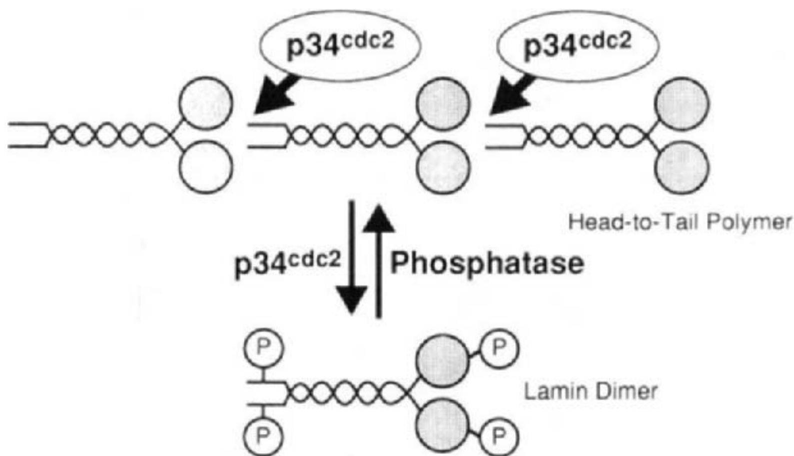

Figure 2. Structural consequences of phosphorylation of lamin head-to-tail polymers by cdc2 kinase. Phosphorylation of *in vitro* reconstituted lamin head-to-tail polymers by cdc2 kinase results in dissassembly into lamin dimers. Subsequent dephosphorylation of such phosphorylated dimers allows the reformation of lamin head-to-tail polymers. These results indicate that mitotic phosphorylation of lamins interferes with longitudinal assembly of lamin dimers. For further discussion see Peter et al., 1991.

Interestingly, a mutation in the amino terminal cdc2 phosphorylation site of chicken lamin B_2 (Ser-16) renders the polymers resistant to disassembly by cdc2 kinase (Peter et al., 1991). The amino terminus has previously been implicated in lamin–lamin interactions (Georgatos et al., 1988) as well as in the formation of cytoplasmic intermediate filaments (Traub and Vorgias, 1983; Geisler and Weber, 1988; Chou et al., 1989), and truncated versions of chicken lamin B_2 lacking the amino terminus fail to assemble into head-to-tail polymers (Heitlinger et al., 1992). Mutations of the second major cdc2 phosphorylation site after the central rod domain of chicken lamin B_2 have no detectable effect on the disassembly of preformed head-to-tail polymers *in vitro* (Peter et al., 1991). However, using a transient transfection assay, Heald and McKeon (1990) found that whereas mutations of the corresponding C-terminal phosphoacceptor site did not render human lamin A resistent to disassembly, they enhanced the effects of N-terminal mutations (Heald and McKeon, 1990). Conceivably, the phosphorylation state of this latter site might control the lateral interactions between head-to-tail polymers. Alternatively, it is possible that *in vivo* phosphorylation of this site might influence interactions between lamins and other proteins, for example, chromatin-associated proteins or components of the nuclear membrane. For instance, a 54-kDa transmembrane protein of the inner nuclear membrane was proposed to serve as a receptor for B-type lamins and was shown to associate with the nuclear lamina in a cell cycle dependent manner (Worman et al., 1988, 1990; Bailer et al., 1991, Simos and Georgatos, 1992). Recent evidence indicates that p54 is phosphorylated by an associated kinase (Simos and Georgatos, 1992) and in addition might be a mitotic substrate of cdc2 kinase (Bailer et al., 1991; Courvalin et al., 1992). These results suggest that the interaction between B-type lamins and p54 are regulated by phosphorylation.

It is interesting to note that the disassembly of preformed lamin head-to-tail polymers by phosphorylation is a reversible process: *de*phosphorylation of phosphorylated lamin dimers by purified phosphatase type 1 results in *re*formation of head-to-tail filaments (Figure 2; Peter et al., 1991). This *in vitro* result is consistent with *in vivo* observations suggesting that dephosphorylation of the nuclear lamins is necessary to allow reformation of the nuclear envelope around the separated chromosomes after mitosis (Pfaller et al., 1991, for review see Nigg, 1992).

V. CONCLUSION

In summary, multiple lines of evidence indicate that mitotically activated cdc2 kinase may trigger mitotic disassembly of the nuclear lamina by acting *directly* on lamin proteins. First, highly purified cdc2 kinase phosphorylates lamins *in vitro* on sites that are phosphorylated during mitosis *in vivo* (Peter et al., 1990; Ward and Kirschner, 1990; Dessev et al., 1991; Lüscher et al., 1991). Second, cdc2 kinase induces lamina disassembly when incubated either with isolated nuclei (Peter et al., 1990; Dessev et al., 1991) or lamin head-to-tail polymers prepared from bacterially expressed lamin proteins (Peter et al., 1991). Third, mutations of the major cdc2 phosphorylation sites on lamins confer resistance to disassembly both *in vivo* (Heald and McKeon, 1990) and *in vitro* (Peter et al., 1991). Finally, genetic evidence obtained from a fission yeast strain expressing chicken lamin B_2 suggests that cdc2 kinase directly phosphorylates lamin proteins (Enoch et al., 1991).

Many of the events occurring at the G1/S and G2/M transitions of the eukaryotic cell cycle may be controlled by a cascade of events initiated by activation of cdc2 kinase. Among all mitotic events, nuclear lamina disassembly represents one of the best characterized downstream events of cdc2 activation. The observation that cdc2 kinase may be directly involved in breakdown of the nuclear lamina suggests a mechanism whereby the eukaryotic cell is able to couple the integrity of the nuclear envelope with the cell cycle.

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DISSECTION OF THE CELL CYCLE USING CELL-FREE EXTRACTS FROM *XENOPUS LAEVIS*

C.C. Ford and H. Lindsay

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ABSTRACT

In the ten years since Lohka and Masui developed extracts from amphibian eggs capable of nuclear assembly, DNA replication, and mitotic chromosome condensation, the use of these and derivative extracts has expanded prodigiously. The relative ease with which these extracts are made from naturally synchronous starting material has provided an exceptional approach to many aspects of the cell cycle. The methods used to generate these extracts are considered first, together with the features that contribute to nuclear construction. A correctly assembled nucleus is essential for S phase, which is the dominant interphase activity of early embryonic cell cycles. Having initiated S phase, mitosis can be delayed under particular circumstances, and it seems to us that the source of the signal delaying mitosis is the replication complexes themselves.

Extracts for studying M phase can be prepared from unfertilized eggs or from interphase cells approaching mitosis, but always in the presence of a calcium-chelating agent. However, free calcium in extracts is present at physiological levels. A calcium-mediated event is required, not only in exit from meiotic metaphase, but also for inactivation of cdc2 kinase during mitosis.

I. INTRODUCTION

The cell cycles that occur during cleavage in amphibian embryos are unusual in that they can be extremely rapid. In *Xenopus* at 21°C, the first cell cycle takes 90 min but subsequent cycles last 35 min each, the embryo accumulating some 4000 cells in seven hr before the cycle slows

and gastrulation proceeds. In order to accommodate these rapid divisions, the somatic cell-size constraints are modified in developing oocytes such that they grow and accumulate components while retaining a G_2 (4C) DNA content. Since the subsequent meiotic divisions are highly asymmetric, the egg inherits these oogenetic stores. Cleavage then runs on these stores, without transcription and with a roughly logarithmic decrease in cell volume until the blastomeres resume a nucleocytoplasmic volume ratio close to that of adult diploid cells. Thus, in comparison to somatic dividing cells, early embryonic blastomeres of amphibians do not grow between divisions, do not require growth factors, and do not require new transcripts to maintain the cyclical progression through interphase and mitosis.

The large size of amphibian eggs and their external fertilization and development makes them particularly convenient for manipulative studies. *Xenopus* has the additional advantages of laying eggs in quantity and essentially on demand. For cell cycle studies, not only can oocytes in isolation be induced to undergo the meiotic divisions but also the unfertilized eggs generated are naturally synchronized in metaphase of meiosis II and can be fertilized or activated synchronously. Using microinjection, it was established that activated egg cytoplasm could induce DNA replication on a wide range of templates while cytoplasms from unfertilized eggs had two special features. These cytoplasms could induce metaphase arrest on injection into blastomeres and could induce meiotic maturation in the absence of hormone on injection into oocytes (Smith and Ecker, 1969; Masui and Markert, 1971). The activities detected were cytostatic factor (CSF) and maturation promoting factor (MPF), respectively. While some purification of each of these activities has been achieved using the microinjection assays (Wu and Gerhart, 1980; Shibuya and Masui, 1989), the development of cell-free extracts provided a convenient assay for two aspects of MPF (nuclear envelope breakdown and chromosome condensation) and allowed an MPF to be purified (Lohka et al., 1988).

In this chapter we will consider the ways in which concentrated extracts from *Xenopus* oocytes and eggs have been prepared and their general properties. In particular, we will focus on how S phase is initiated, how the timing of mitosis may depend on the completion of S phase, and whether calcium-mediated events are required during cdc2 kinase activation and inactivation through M phase.

II. PREPARATION OF EXTRACTS

A. Obtaining Cells

Two cell types—the fully grown oocyte and the unfertilized egg—are the most common starting materials for making extracts from *Xenopus*. Animals can be maintained relatively easily, being fed twice a week with either raw meat or dry food pellet (see Wu and Gerhart, 1992, for details of both maintenance and rearing).

To obtain eggs, adult females are injected with human chorionic gonadotrophin (550–700 units per animal depending on size), 15 to 17 hr before the eggs are required. The advantage of a single hormone injection is that eggs tend to be shed in a shorter burst than is obtained with a two-injection regime. If the animals are being fed a diet of dry pellet, it is advantageous to leave the animals in water for several hours after injection before transferring them to the tanks in which they will lay. This is because some defecation or regurgitation of food can occur as an early response to injection and consequently fragments of pelleted food are present with the eggs. These fragments are not easily separated from the eggs by washing. Frogs are left at 21°C in either saline tap water (110 mM NaCl in tapwater) or high salt Barth X (Barth X made 110 mM in NaCl; Blow and Laskey, 1986; see Leno and Laskey, 1992) to shed eggs. Under these conditions, shed eggs remain fertilizable for considerable time (Wolf and Hedrick, 1971).

Egg quality is crucial for obtaining functional extracts. Any batches of eggs where the eggs have been laid in strings of jelly are not used, as these eggs are often misshapen. Such batches occur more frequently when the female has been left for a long period since her last egg laying. Consequently, we induce laying on a 3-month cycle. This may reduce the quantity of eggs laid. Eggs from each female are dejellied separately (in 5 mM dithiothreitol, 110 mM NaCl, 20 mM Tris pH 8.5, see Leno and Laskey, 1992, for alternatives), rinsed three times in saline tap water, and held in this medium for inspection. Poor batches of eggs show instability in dejellying and have significant numbers of white necrotic cells (>10%). Good batches of eggs remain stably unactivated through this procedure and have a uniformly pigmented animal hemisphere. Eggs that have remained in saline solutions for long periods (> 5 hr) develop white circular patches within the animal hemisphere (distinct from the *white spot* of the unfertilized egg), but such batches of eggs are rare when

the laying regime described above is used. The few necrotic eggs that may be present in an otherwise *good* batch of eggs are removed prior to two washes in distilled water and transferred to extraction buffer on ice.

Oocytes are isolated by collagenase treatment of dissected ovary fragments (Leno and Laskey, 1992). Females can be injected with 50 iu follicle-stimulating hormone (Folligon) 1 week prior to use, to increase the number of large (Stage V and VI; Dumont, 1972) oocytes. However, in our hands, this occasionally leads to a low level of spontaneous maturation. The lobes of isolated ovary are opened, cut into pieces, and washed in Barth X (or OR2) before transfer to medium containing collagenase (Smith et al., 1992; Leno and Laskey, 1992; Patrick et al., 1989). Treatment time varies with each ovary and in practice we agitate the ovary fragments and remove separated oocytes as they are released. These are washed extensively in Barth X, and stage V and VI oocytes are selected. As with egg selection, only undamaged cells with uniform animal hemisphere pigmentation are taken.

B. Ground Rules

The pioneering work of Lohka and Masui (1983) using eggs of *Rana pipiens* and Fred Lohka's subsequent adaptation of his protocol for *Xenopus* eggs (Lohka and Maller, 1985) provided the ground rules for making active extracts from amphibian eggs. Three rules emerged from their work. First, extracts should be made using packed eggs with a minimum of surrounding buffer. This ensures high protein concentrations (>30 mg/ml) in the resulting cytoplasmic supernatants. Second, eggs should be crushed by centrifugation, not by homogenization. Protein synthesis is severely reduced in extracts prepared by homogenization compared to spin-crushed extracts (T. Patrick, personal communication). Release of highly phosphorylated yolk proteins (phosvitin and lipovitellin) during homogenization may also be detrimental (e.g., for chromatin assembly; Wolffe and Schild, 1992). Third, both the soluble (high-speed) supernatant and the *fluffy* membrane fraction are essential for nuclear formation from demembranated sperm heads. It is now clear that an initial phase of nucleoplasmin-mediated chromatin decondensation of sperm heads can be achieved in the absence of a membrane fraction (Philpott et al., 1991) but for maximal decondensation, the membrane fraction is essential.

C. Interphase Extracts

The original procedure of Lohka and Masui used *Rana* eggs taken after 1 hr incubation at 19°C following electrical activation. Eggs were washed in ice cold buffer (see Table I) and collected in centrifuge tubes. After centrifugation at 9000 ×g for 15 or 30 min, the resultant extract had been fractionated into four layers—yolk, heavy cytoplasm, a more dilute layer, and a lipid pellicle. They took the heavy cytoplasmic layer and recentrifuged under the same conditions to remove most of the pigment. These extracts were used immediately. Subsequently, Lohka and Maller (1985) modified the conditions (primarily of the extraction buffer, Table 1) for use with *Xenopus* eggs. Sucrose was omitted from this buffer (it can depress translation, see Protein Synthesis), and cytochalasin B was added to the first cytoplasmic supernatant, presumably to minimize gelation. Protease inhibitors (PMSF and leupeptin) were added to the starting buffer, though later modifications of the buffer avoid PMSF as it too can depress translation in fresh extracts.

Several laboratories have made further modifications to the buffer and/or preparation procedure (Table I), depending on the emphasis of the questions analyzed. Blow and Laskey (1986) found conditions for recovering replication activity from frozen extracts, which involved direct freezing into liquid nitrogen in small (15 µl) aliquots after addition of glycerol (2%–10%) with or without 2 mM ATP (Leno and Laskey, 1992). Newport (1987) introduced a packing spin (30 sec, 100 ×g) to reduce the amount of buffer present in the subsequent crushing spin. In our experience, this is preferable when small (2 ml or less) tubes are used for extract preparation, though it is not essential when 5-ml tubes are used, presumably because the larger volume of eggs in a 5-ml tube pack more tightly under gravity. Murray and Kirschner (1989) further eliminated buffer during the packing spin by adding the eggs to tubes containing Versilube 50, which is intermediate in density between eggs and buffer (Murray 1992). Such extracts undergo multiple rounds of cell cycle events (5 cycles in 5 hr at 22°C, Hutchison et al., 1988) with or without a packing spin (Murray and Kirschner, 1989; Hutchison et al., 1988).

D. Unfertilized Egg Extracts (CSF Extracts)

The unfertilized *Xenopus* egg is arrested at metaphase of the second meiotic division and held there by a Ca^{2+}-sensitive activity called *cytostatic factor* (CSF) (Masui and Markert, 1971). Fertilization or activation

Table 1. Extraction Buffers

Component	*Lohka Masui (1983) (for Rana)*	*Lohka Maller (1985)*	*Blow Laskey (1986)*	*Newport (1987)*	*Hutchison Cox Ford (1988)*	*Murray Kirschner (1989)*	*Almouzni Mechali (1988)*	*Felix et al. (1989)*
KCl (mM)	200	100	50	50	110	100	70 mM K Acetate	100 mM K Acetate
Hepes (mM)	100 mM Tris	20	50	—	20	10	20	—
(pH)	(7.5)	(7.5)	(7.4)		(7.5)	(7.7)	(7.5)	—
$MgCl_2$ (mM)	1.5	5.0	5.0	2.5	5.0	1.0	—	2.5 mM Mg acetate
DTT (mM)	[a]2.0	2.0	[a]2.0	1.0	[a]2.0	—	1.0	1.0
$CaCl_2$ (mM)	—	—	—	—	—	0.1	—	—
EGTA (mM)	—	[b]10	—		—	[b]5	—	60
Sucrose (mM)	250	—	—	250	—	50	5%	250
Protease inhibitors	—	0.3 mM PMSF 3 µg/ml leupeptin	3 µg/ml leupeptin	—	[c]80 KU/ml aprotinin	[c]10 µg/ml leupeptin chymostatin& pepstatin	—	—
Cytochalasin B	—	[c]50 µg/ml	10 µg/ml	5 µg/ml	[c]50 µg/ml	[c]10 µg/ml	—	5 µg/ml
Other additions	—	—	[d]10% glycerol 2 MM ATP	100 µg/ml cycloheximide	—	[d]200 mM sucrose	—	—
Packing	—	—	—	+	—	+	—	—

Notes: [a]β-mercaptoethanol
[b]only added for preparing CSF extracts
[c]added to first cytoplasmic supernatant
[d]added prior to freezing

stimulates a transient increase in the intracellular free calcium concentration. Because of this sensitivity to calcium, using unfertilized eggs as starting material requires special steps to ensure that the resulting extract retains properties of an arrested meiotic metaphase cytoplasm. Since spontaneous activation can occur even in high-salt solutions containing EGTA, freshly laid eggs, squeezed from the female, are likely to give the highest proportion of unactivated eggs. These can be accumulated over several hours by stabilizing each batch of eggs using the "hardening" procedure of Wangh (1989). Two other differences in protocol compared to interphase extracts are crucial. First, a Ca^{2+}-chelating agent (usually EGTA) should be present in the extraction buffer. Second, *Xenopus* eggs should not be subjected to cold or osmotic shock, both of which increase the chances of spontaneous activation. A step-by-step protocol for preparing such extracts (called CSF extracts because they are held in meiotic metaphase by retention of CSF activity) is described by Murray (1992).

E. Oocyte Extracts

In order to make extracts from oocytes by spin crushing rather than homogenization, it is necessary to collagenase treat the oocytes (see Obtaining Cells above) to remove much of the follicular layers. Once this is done, extracts are prepared in the same way as interphase egg extracts (Cox and Leno, 1990; Leno and Laskey, 1992).

F. Fractionation of Low-Speed Extracts

A commonly used, simple first fractionation step is to centrifuge the low-speed supernatant (LSS) at 100,000 ×g to generate three fractions—an orange gelatinous ribosomal pellet, a membrane fraction, and a high-speed supernatant (HSS) fraction (Lohka and Masui, 1984; Newport and Spann, 1987; Sheehan et al., 1988). The high protein content of extracts makes them viscous, and consequently recentrifugation of the final supernatant is sometimes needed to ensure elimination of membranes (Leno and Laskey, 1992). The high-speed supernatants obtained are incapable of nuclear formation without readdition of the membrane fraction, and protein synthesis is almost completely eliminated (see Protein Synthesis below). *Mitotic* membranes from CSF extracts have been further fractionated on sucrose step gradients into *light* and *heavy* fractions (Wilson and Newport, 1988). Only the *light* membrane fraction is active in nuclear envelope assembly. Vigers and Lohka (1991) have

also separated membranes into two fractions by differential centrifugation. In this case both fractions were required for formation of a functional nuclear membrane.

Recently, Toyoda and Wolffe (1992) have fractionated oocyte and egg low-speed extracts by ammonium sulphate precipitation to generate fractions capable of accurate RNA polymerase II directed transcription. An alternative powerful approach to fractionating extracts is to use selective affinity matrices to functionally deplete or selectively remove specific components (e.g., lamins: Meier et al., 1991; Newport et al., 1990; Eg5 protein: Sawin et al., 1992; cdc2 protein kinase: Solomon et al., 1990). Though affinity matrices may well remove components other than the expected target molecule, this approach is powerful when coupled to readdition of the target component alone.

III. GENERAL PROPERTIES OF EXTRACTS

A. Chromatin Assembly

Both oocyte and egg extracts are capable of converting added purified DNA into chromatin (Laskey et al., 1977; reviewed Reeves, 1992), a property retained by high-speed supernatants (HSS) devoid of membranes. "Nucleosomelike" particles are not efficiently formed on single-stranded DNA templates (Almouzni et al., 1990). However, when complementary strand DNA synthesis occurs, as it does in both oocyte and egg extracts (Mechali and Harland, 1982; Blow and Laskey, 1986; Cox and Leno, 1990), the newly formed double-stranded DNA is more efficiently assembled into chromatin than nonreplicating double-stranded DNA (Almouzni et al., 1990). Supercoiled pBR322 DNA takes some 4 hr to be assembled into chromatin in either egg or oocyte HSS (Almouzni and Mechali, 1988) while some 80% of DNA added to egg LSS can be incorporated into chromatin in 40 min (Newport, 1987). Though this is significantly faster, the concentration of DNA present in the extract was some 10-fold less.

Normally, of course, one substrate an egg has to convert into a pronucleus is the tightly packaged sperm head. *In vivo* the demembranated sperm head undergoes an initial rapid, but partial, decondensation followed by a slower, membrane-dependent decondensation. *In vitro* these two stages can be conveniently separated by high-speed centrifugation (Lohka and Masui, 1984). Philpott et al. (1991) have shown, by immunodepletion of nucleoplasmin followed by readdition of purified

nucleoplasmin, that the rapid initial decondensation is dependent on this protein. Indeed purified nucleoplasmin alone induces a similar rate and extent of sperm chromatin expansion as an undepleted egg extract (Philpott et al., 1991). When histones H_2A and H_2B are also present with the nucleoplasmin, remodeling of the sperm chromatin occurs, forming nucleosomes (Philpott and Leno, 1992). Sperm chromatin injected into oocytes or added to oocyte extracts also undergo at least the first stage of decondensation (Cox and Leno, 1990). Thus by these two parameters (chromatin assembly and stage I decondensation) there is little difference between oocyte and egg extract, despite their diametrically opposed capacities for transcription and replication observed *in vivo*.

B. Nuclear Envelope Assembly

The egg membrane fraction is essential for nuclear envelope formation and the second phase of chromatin decondensation. Thus only low-speed egg extracts (or high-speed extracts to which egg membranes are readded) can form fully decondensed, replication-competent, nuclei. Oocyte membrane fractions cannot substitute for egg membranes, and oocyte supernatant cannot substitute for egg supernatant in mixing experiments (Cox and Leno, 1990). Assembly requires egg supernatant components as well as two distinct membrane vesicle fractions (Vigers and Lohka, 1991). One fraction associates with chromatin in an ATP-independent manner, but membrane fusion requires both the second fraction and ATP (Vigers and Lohka, 1991).

Surprisingly, lamin LIII, which is normally polymerized into a network subjacent to the nuclear membrane during nuclear assembly or reformation, is not essential for nuclear membrane assembly or chromatin decondensation in *Xenopus* egg extracts (Newport et al., 1990; Meier et al., 1991). In contrast, in somatic cell mitotic extracts (Burke and Gerace, 1986) and mitotic cells (Benavente and Krohne, 1986) undergoing nuclear assembly on exit from mitosis, functional depletion of lamins prevents chromatin decondensation in both situations, though nuclear membrane assembly is prevented only in the mitotic extracts. The difference in membrane assembly observed with immunodepleted mitotic extracts compared to antibody injected mitotic cells may reflect the different protocols used, in that the injected antibody may not have sequestered an *early*-attaching lamin population sufficient to allow formation of a nuclear membrane (Benavente, 1991). However, in contrast to the results in egg extracts, this is still not sufficient to allow chromatin

decondensation. Clearly, in somatic cells decondensation is tightly coupled to polymerization of a complete lamina.

This coupling may be critical for the spatial order and extent of decondensation in a reforming somatic interphase nucleus. In *Xenopus* extracts, decondensation is uncoupled from polymerization of the lamina. Early embryonic nuclei contain uniformly decondensed chromatin and are transcriptionally inactive. Thus differential control of decondensation may be unimportant at this stage. Proteins other than lamins are involved in targeting membrane vesicles to chromatin (Wilson and Newport, 1988; Vigers and Lohka, 1991). It may be that particular proteins of the early embryonic nucleus usurp the role of lamins in stimulating global chromatin decondensation, though it is also possible, as suggested by Meier et al. (1991), that the high levels of topoisomerase II in eggs is sufficient to bypass the requirement for a lamina in decondensation of embryonic chromatin.

C. Chromatin Condensation

Two stages of chromatin condensation can be distinguished in low-speed egg extracts in which sperm pronuclei have formed. In the first the initially uniformly decondensed chromatin becomes nonuniformly aggregated within an expanding nuclear envelope, and usually chromatin fibers are observed (Hutchison et al., 1988, 1989). This level of condensation is independent of MPF activation, since it occurs in interphase extracts arrested by inhibiting protein synthesis. Indeed, in cycloheximide arrested extracts, the chromatin often condenses to a compact *cigar* or S shape reminiscent of an early decondensing sperm head. Further condensation occurs on addition of mitotic extract (Lohka and Maller, 1985; Newport and Spann, 1987) or in interphase extracts allowed to synthesize proteins so that MPF activates automatically (Hutchison et al., 1988). Completion of replication is not required for either stage of condensation in egg extracts (Hutchison et al., 1989). However, there is suggestive evidence that a normally polymerized lamina is a prerequisite for the first phase of condensation (Meier et al., 1991; I. Kill personal communication).

D. Protein Synthesis

Both oocyte and egg low-speed extracts can synthesize proteins on endogenous templates at close to the rate observed *in vivo* when used immediately after preparation (Patrick et al., 1989; Matthews and Coleman, 1991). Translational activity is usually depressed substantially after

freezing, though Murray (1992) reports that addition of 200 mM sucrose (final concentration) depresses translation in fresh extracts, but no further loss of capacity is observed after freezing. Translation in thawed extracts can be increased by supplementing this reaction with small quantities of reticulocyte lysate S100 and creatine phosphate (Mathews and Colman, 1991). Progesterone matured oocyte extracts show increased rates of translation compared to oocyte extracts (44%; Patrick et al., 1989) in line with the estimated twofold to threefold increase *in vivo* (Wasserman et al., 1982). Some 25 μg protein (about 10% of the methionine pool) can be synthesized per milliliter of extract per hour at 21°C in egg extracts that can be increased up to sixfold by supplementation (Patrick et al., 1989; Mathews and Colman, 1991). In our hands, unsupplemented extracts incorporating more than 3% of the methionine pool per hour undergo periodic mitotic events and can continue incorporation approximately linearly for 4 hr (Hutchison et al., 1988; unpublished observations). Extracts supporting high levels of translation (>10% methionine pool/hr) do not sustain these levels for much more than an hour (see Mathews and Colman [1991] for comparison of kinetics for different extract protocols and following supplementation).

The pattern of endogenous translation (analyzed on 1D gels) differs between oocyte and egg extracts, reflecting qualitatively the difference in patterns observed *in vivo* (Patrick et al., 1989). Added synthetic mRNA can be translated in both types of extract, though a given mRNA may not be equally effective in both, or indeed translated at all (Murray and Kirschner, 1989; Patrick et al., 1989; Mathews and Colman, 1991; Norbury et al., 1991). Changes in polyadenylation and recruitment and withdrawal from the polysome pool occur between oocyte, egg, and early embryo; and thus the 5′ and 3′ untranslated regions of a given mRNA will have a substantial impact on its translatability in extracts from different stages (see Standart, 1992; Wickens, 1992 for recent reviews of this area). In practice, the probability that a given coding sequence can be translated can be increased by insertion between the *Xenopus* β-globin 5′ and 3′ flanking sequences (Krieg and Melton, 1984; Wormington, 1992; Mathews and Colman, 1991).

IV. DNA REPLICATION

A. Changes between Oocyte and Egg

Microinjection experiments established that a qualitative change occurred between the oocyte and egg in the capacity to initiate DNA

replication on double-stranded DNA or nuclei (Gurdon, 1967; Harland and Laskey, 1980; reviewed by Ford, 1985). The failure of oocytes to initiate replication of these substrates is apparent despite the presence of stores of components necessary for both chromatin assembly and DNA synthesis. Indeed replication can continue in previously initiated S phase nuclei, and single-stranded templates are converted to a double-stranded form, both *in vivo* and in extracts (Gurdon, 1968; Ford and Woodland, 1975; Cortese et al., 1980; Cox and Leno, 1990). Thus it appears that many of the expected activities for replication are present and active in oocytes, yet they fail to initiate S phase.

Attempts to complement oocyte extracts by combining oocyte and egg fractions *in vitro* (Cox and Leno, 1990) showed that neither the membrane fraction nor the supernatant from oocyte extracts could replace the corresponding fraction from eggs. This suggests that changes in the membrane fraction are required to generate an appropriately assembled nuclear template for S phase as well as changes in the soluble fraction. The latter presumably include the synthesis or activation of specific initiation factors. As indicated above (see Protein Synthesis) changes in polyadenylation state of mRNAs leads to an altered profile of protein synthesis during maturation. cDNA clones for some of these mRNAs have been isolated (Dworkin et al., 1985; Paris and Philippe, 1990). In particular, cdk2 protein (formerly EgI, Paris et al., 1991) increases substantially in amount between oocyte and egg and has been implicated in the initiation of S phase.

B. Initiating S Phase

An unusual feature of the cell cycle in *Xenopus* eggs and extracts made from them is that protein synthesis is not required for first S phase, though it is required for mitosis (Harland and Laskey, 1980; Miake-Lye et al., 1983; Blow and Laskey, 1986; Hutchison et al., 1988). Thus, in comparison to cell cycles of somatic cells or yeast, the egg is committed to S phase from the moment of fertilization and exit from mitosis. Indeed the vegetal region of the unfertilized egg is capable of supporting nuclear DNA synthesis, presumably reflecting a localization of MPF and/or CSF to the animal hemisphere (Gurdon, 1968). Since there is no requirement for transcription, translation, or growth factors for the onset of S phase following meiosis or the early mitotic divisions, the egg appears to lack an analog of the restriction point of somatic cells or the start control point in yeasts (reviewed in Norbury and Nurse, 1992). The necessary post-start pre-S events occur during maturation. Somatic cells lacking a G_1

might achieve this through a translational control circuit coupling polyadenylation and polysome recruitment to the preceding mitosis.

A second peculiarity of S phase in early *Xenopus* embryos is that it is extremely short. This is achieved by synchronous initiation at many sites at the start of S (Callan, 1972). It appears that initiation events in different regions of chromatin are not staggered as they are in the extended S phases of somatic cells. The absence of this differentiation may mean that all sites are constructed and initiated in a similar manner, which would help in their dissection. By using short pulses of biotin-11-dUTP incorporation into S-phase sperm pronuclei in egg extracts, Mills et al. (1989) detected foci of biotin incorporation throughout the depth of the nucleus, throughout S phase. They estimate that each focus represents a cluster of more than 300 replication forks and argue that the sites remain fixed throughout S phase. The topological constraints implied by such a system require not only a highly ordered construction of replication foci, but also a release mechanism to allow the insertion of the final nucleotides on each daughter strand of converging replication forks. Perhaps here is a role for the unusual repair and joining reaction that is present in egg extracts but not oocytes (Pfeiffer and Vielmetter, 1988).

Both PCNA (DNA polymerase δ auxilliary protein) and DNA polymerase α have a punctate distribution in replicating sperm pronuclei consistent with a picture of replication foci and the distribution of both antigens changes in a nuclear autonomous manner as replication terminates (Hutchison and Kill, 1989). In the presence of aphidicolin (which inhibits replication), both PCNA and DNA polymerase α become chromatin associated and remain so even if nuclear envelope breakdown and lamin dispersal are induced by MPF activation or addition. Thus replication complexes, once formed, are extremely stable in the presence of aphidicolin to changing cytoplasmic state, but apparently disperse when replication is completed. The ability of aphidicolin-blocked nuclei, formed in egg extract, to continue replication in oocyte extracts in the absence of aphidicolin implies that aphidicolin blocks after initiation events are completed and prevents continuation and termination (Cox and Leno, 1990).

What events are necessary for the initiation of S phase (for the formation of an aphidicolin-sensitive nucleus), and how are they regulated? We envisage three aspects to this problem. First, there are the components (DNA polymerases, ligases, etc.) required in replication complexes. Second, nuclear organization must be such that formation of replication-competent complexes occurs. This requires chromatin

decondensation and at least lamin III, as nuclei formed in the absence of lamin III fail to replicate (Newport et al., 1990; Meier et al., 1991). In the absence of a functional lamina, PCNA associates with nuclei but fails to distribute with the chromatin in the way it does in S-phase nuclei, which suggests that lamin III provides sites, or directs components to sites, for replication complex assembly (Meier et al., 1991). Third, there may be regulatory events to activate the sites of replication, the components being assembled, and/or the replication complexes once formed.

The most direct evidence for such regulatory events in *Xenopus* extracts comes from analyzing the effects of depleting cdc2-like proteins (Blow and Nurse, 1990; Fang and Newport, 1991). Blow and Nurse showed that depletion of cdc2-like proteins from egg extracts using either $p13^{suc1}$ beads or a polyclonal $p34^{cdc2}$ antibody inhibited replication, though nuclear membranes formed and elongation of preinitiated nuclei could occur. This suggests that a cdc2-like protein is required for events up to formation of aphidicolin-sensitive replication complexes. The event(s) occur early in nuclear formation since depletion 15 min after addition of demembranated sperm heads failed to inhibit replication (Blow and Nurse, 1990). Fang and Newport (1991) using polyclonal antisera raised against distinct C-terminal peptides of cdc2A (*Xenopus* $p34^{cdc2}$ homologue) and cdc2B (now called cdk2, but originally Eg1, Paris et al., 1991), showed that depletion of about 90% of cdk2 inhibited replication while depletion of 75% of $p34^{cdc2}$ did not, though it did prevent mitosis. This suggests that cdk2, but not cdc2, is essential for S phase. Both reports indicate that replication can be rescued by addition of proteins eluted from the $p13^{suc1}$ beads, a fraction enriched in cdc2-like proteins.

We have used both $p13^{suc1}$ beads and a polyclonal antiserum to cdk2 to deplete egg extracts (Chevalier et al., 1995). In our hands, the cdk2 antiserum removes much of the cdk2, but also removes some cdc2 while $p13^{suc1}$ beads remove all PSTAIR reactive bands extensively. Both procedures reduce the labeling index of template sperm pronuclei from greater than 90% in controls to 10% to 40% after depletion. This is consistent with inhibition of initiation, since initiation is autonomous to each nucleus; those nuclei that do manage to initiate then replicate effectively. Surprisingly, in attempts to rescue initiation we found that either cdk2 or cdc2 proteins, readded to the depleted extracts through translation of their respective synthetic mRNAs, increased the labeling index threefold to sixfold. We suggest that, under circumstances where new protein synthesis occurs, either $p32^{cdk2}$ or $p34^{cdc2}$ can fulfill the

requirement for efficient S-phase initiation. We presume that, in Fang and Newport's experiments where $p34^{cdc2}$ remains in the extract following $p32^{cdk2}$ depletion, $p34^{cdc2}$ is not in an appropriate compartment to substitute for cdk2.

How cdc2-like proteins contribute to initiation of S phase is not understood. As *Xenopus* eggs do not show a *restriction point* using the perturbations that expose it in somatic cells, the implication of the depletion experiments above is that cdc2-like proteins are necessary directly for S-phase initiation in addition to other functions they may have at the 'restriction' point. Several recent reports indicate that SV40 replication can be stimulated by cdc2-like proteins (including purified cdc2-cyclin B) and that RPA is regulated by phosphorylation (D'Urso et al., 1990; Fotedar and Roberts, 1992; Dutta and Stillman, 1992). However SV40 replication *in vitro* occurs without nuclear formation. It is thus possible in *Xenopus* extracts that cdc2-like proteins are required for generating appropriate nuclear sites for replication complex formation in addition to stimulating complex formation and activation.

In an analysis of the role of protein phosphatase I (PPI) during the cell cycle of egg extracts, Walker et al. (1992) found that DNA synthesis was inhibited by the presence of Inh2, a specific PPI inhibitor. In this situation chromatin decondensation did not occur, even though nuclear membranes formed. This suggests that PPI is necessary for decondensation to produce a nucleus structurally competent to enter S phase.

A further protein that may be necessary for generating an appropriate nuclear conformation for replication is the RCCI protein. A temperature-sensitive mutation of the RCCI gene in hamster cells (tsBN2) prevents entry into S phase when isoleucine-deprived G_1 cells are released from deprivation at the restrictive temperature (Nishimoto et al., 1978). In *Xenopus* extracts depleted of RCC1 protein, nuclei with decondensed chromatin form but do not undergo DNA replication (Dasso et al., 1992). The protein can associate with chromatin in the absence of a nuclear membrane and is present in nuclei at high concentrations (one molecule per 210 bp of DNA, Dasso et al., 1992). The role of RCCI in S phase is not understood, though it is not required for second-strand synthesis on single-stranded templates. However, loss of RCCI during S phase inhibits replication and at the same time advances mitosis in tsBN2 cells at the restrictive temperature (Nishimoto et al., 1978; Nishitani et al., 1991). RCCI protein is found complexed with a *ras*-related nuclear protein (*ran*) in HeLa cells and specifically catalyzes exchange of guanine nucleotides on the *ran* protein (Bischoff and Ponstingl, 1991a,b). This potentially

provides a signal that might delay mitosis during replication (Roberge, 1992) but might equally be necessary to initiate and sustain S phase and consequently delay mitosis. In tsBN2 cells, it is not clear whether premature mitosis inhibits replication or premature cessation of S phase advances mitosis.

C. Dependence of M Phase on Prior S phase

In a *normal* cell cycle, M phase not only follows S phase but does not occur if S phase is arrested. The dependency of M phase on completion of S phase implies either that the replicated nucleus contributes a component(s) for M phase (a substrate in a pathway, Hartwell et al., 1974) or that a regulatory network not itself essential for M phase can monitor S phase and delay M phase until S phase is completed (a G_2 checkpoint, Hartwell and Weinert, 1989). At first sight *Xenopus* eggs appear to have lost this dependency since enucleate egg fragments and aphidicolin-treated eggs continue to show periodic surface contractions and periodic MPF activation (Hara et al., 1980; Newport and Kirschner, 1984). However, in cycling egg extracts, addition of aphidicolin can delay mitosis and does so in a manner dependent on the concentration of nuclei (Dasso and Newport, 1990; Hutchison et al., 1989). At low nuclear concentrations, typical of the nuclear/cytoplasmic volume ratios of early cleavage embryos, aphidicolin fails to delay M phase in extracts. These results suggest that each replication-blocked nucleus can generate a signal, the concentration of which (and hence effectiveness) depends on the number of nuclei in a given cytoplasmic volume.

In the yeast *Schizosaccheromyces pombe*, several genes (cdc25, wee1, mik1, nim1) have been identified, whose products are involved in regulating the timing of mitosis (reviewed in Roberge, 1992). $p34^{cdc2}$ activation normally requires the product of the cdc25 gene, $p80^{cdc25}$, a tyrosine phosphatase that directly dephosphorylates tyrosine 15 (Y15). wee1 and mik1 gene products negatively regulate mitosis and probably do so by phosphorylating Y15 (reviewed by Millar and Russell, 1992). When replication is blocked in *S. pombe* by hydroxyurea, mitosis is delayed through a pathway that includes $p80^{cdc25}$ (Enoch and Nurse, 1990).

Xenopus CSF extracts released by Ca^{2+} addition and then arrested with aphidicolin and sperm pronuclei show continued accumulation of $p34^{cdc2}$ containing phosphotyrosine (Kumagai and Dunphy, 1991), which suggests that mitosis is delayed by inactivation of a cdc25-like activity or

activation of a tyrosine kinase. Indeed, the delay to mitosis can be overcome by addition of a *Drosophila* homologue of yeast $p80^{cdc25}$ (Kumagai and Dunphy, 1991). A tyrosine kinase activity that phosphorylates *Xenopus* $p34^{cdc2}$ is high in interphase and reduced in mitosis, but remains high in aphidicolin arrested extracts (Smythe and Newport, 1992). Caffeine and okadaic acid, each of which overcomes aphidicolin-induced arrest, both induce a drop in tyrosine kinase activity (Smythe and Newport, 1992). These results suggest that inhibition of replication by aphidicolin leads to an altered balance of tyrosine kinase and phosphatase and accumulation of inactive, tyrosine phosphorylated $p34^{cdc2}$.

There are two other ways, at present, by which aphidicolin-induced delay to mitosis can be circumvented. If cyclin A synthesis is selectively eliminated or if protein phosphatase I is inhibited using inhibitor-2, activation of cyclin-B kinase is advanced (Walker and Maller, 1991; Walker et al., 1992). These treatments might be uncoupling the same or parallel pathways affecting $p34^{cdc2}$ activation. In addition, cyclin-A cdc2 kinase activity can also activate cyclin-B cdc2 kinase measured in the absence of nuclei (Devault et al., 1992). In these experiments, a human cyclin A was used at some 10 times the concentration of *Xenopus* cyclin A detected in the first cell cycle in eggs (Kobayashi et al., 1991). Walker and Maller (1991) observed that, in the absence of nuclei, removal of cyclin-A mRNA did not alter the timing of H_1 kinase activation. Thus the influence cyclin A has on timing mitosis may depend not only on its concentration, but on whether or not nuclei are present. The distribution of cyclin A between nucleus and cytoplasm in *Xenopus* eggs is not known, but in syncytial *Drosophila* embryos, it is detected in both compartments in interphase (Maldonado-Codina and Glover, 1992). The overall balance of influence of cyclin-A kinase may reflect the local concentration and substrates available in different compartments. A further complication arises from the observation that in syncytial *Drosophila* embryos, cyclin A is present through mitosis and into the next interphase (Maldonado-Cordina and Glover, 1992). The implication in *Xenopus* would be that not all the cyclin A is or needs to be degraded to enter interphase and indeed higher levels of cyclin A are present 30 min after activation than in unfertilized eggs (Kobayashi et al., 1991). Different batches of eggs and different activation conditions might contribute to the level of cyclin A inherited at the start of the first cell cycle and might thus account for variation between extracts (Devault et al., 1992).

Mitotic events in *Xenopus* eggs and extracts can occur even in the absence of nuclei. Thus, in this situation at least, nuclear material is not

a necessary substrate for activation of M-phase kinase nor does replicated DNA provide an activator. How then does an extract register the presence of an appropriate number of nuclei arrested during replication and delay mitosis? Aphidicolin-blocked nuclei fail to replicate, contain very stable replication complexes, and delay mitosis (see Initiating S Phase above). In experiments designed to assess the effect of camptothecin, a specific inhibitor of topoisomerase I, on DNA replication, we found that camptothecin failed to block replication. Some 85% of control levels of semiconservative replication occurred (Hynds, 1991; Hynds and Ford, in preparation). However, despite near control levels of replication, mitosis was delayed in a caffeine-sensitive manner. In the presence of camptothecin, DNA replication initially occurred rapidly, but at later times (when control nuclei had completed S phase), low levels of biotin-11-dUTP incorporation were still detected, DNA polymerase α remained chromatin associated, and nuclear envelope breakdown was delayed (Figure 1). We suggest that camptothecin blocks normal termination of replication. The continued presence of chromatin-associated DNA polymerase α, like the retention of replication antigens on chromatin in aphidicolin-treated extracts, could imply that the presence of replication complexes generates a signal delaying mitosis. A signal delaying mitosis might be produced in proportion to the number of replication complexes formed and disappear when replication terminates.

On this basis, nuclei in egg extracts can only influence the timing of mitosis if they are present in sufficient number and if they are in S phase (i.e., have replication complexes). Serendipitously, we have obtained several fresh egg extracts that failed to undergo S phase in the first cycle, judged by the absence of biotin-11-dUTP incorporation, despite formation of apparently normal nuclei with lamina. In these extracts, mitosis occurred and was not delayed by the presence of either aphidicolin or camptothecin (Hynds and Ford, unpublished observation). Our interpretation is that in the absence of replication no replication complexes are formed and no signal to delay mitosis is generated.

In extracts where DNA replication occurs, addition of increasing numbers of nuclei extends the period over which DNA synthesis occurs and delays mitosis (Dasso and Newport, 1990). Blow and Watson (1987) have shown that in any one nucleus the duration of DNA synthesis (S phase) is rather constant at 30 to 40 minutes, but that different nuclei initiate S phase at different times. Thus at high nuclear concentrations, asychrony of initiating replication could maintain the number of repli-

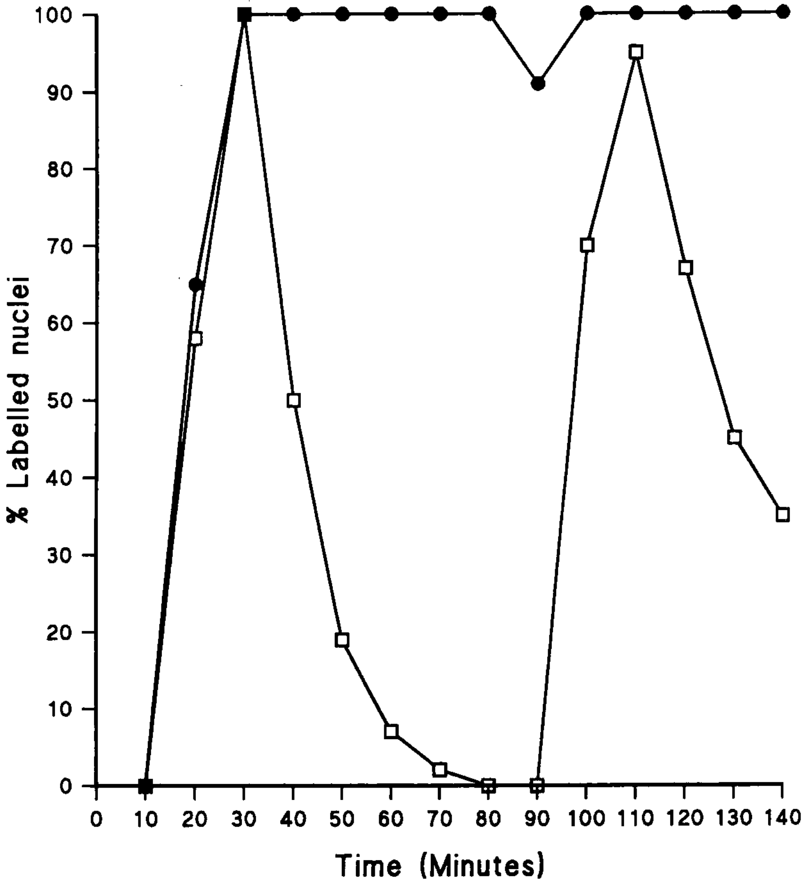

Figure 1. Camptothecin delays exit from S phase and delays mitosis when added to egg extracts. The graph shows the proportion of labeled nuclei detected after successive ten-minute pulses with biotin-11-dUTP in an extract containing either 100 μM camptothecin and 1% DMSO (closed circles) or 1% DMSO alone (open squares) in addition to sperm pronuclei (10^3/μl) using procedures as in Hutchison et al. (1988). The proportion of nuclei in S phase remains high in the presence of camptothecin, though the intensity of labeling dropped after sixty minutes. Mitosis, which occurred at 80 to 90 minutes in the control incubation, failed to occur in the extract containing camptothecin.

cation complexes at a level sufficient to prevent mitosis. Addition of aphidicolin to extracts with nuclei traps each nucleus with replication complexes formed, thus synchronizing the population of nuclei. Thus the number of nuclei required to produce a given delay in mitosis is much less in the presence of aphidicolin than in its absence, as shown by Dasso and Newport (1990).

If the act of replication generates a negative influence on mitotic entry, termination of replication should remove this influence. Using frozen extracts, where nuclear assembly is slower than in fresh extracts, Blow and Watson have shown that once a nucleus initiates replication, the DNA content doubles during the period of S phase. However in the fresh extracts we have used (Hutchison et al., 1988, 1989), measurement of the amount of DNA synthesis in the first S phase together with the labeling index (routinely 90%–100%) indicates that each nucleus replicates only some 35% of its DNA content. S phase terminates around 60 min from the start of incubation, some 20 to 30 min before nuclear envelope breakdown (NEBD, Hutchison and Kill, 1989). While the failure to replicate all the DNA remains to be explained, the synchronous termination of replication in many nuclei suggests replication from any one initiation site is limited to a domain irrespective of whether a neighboring domain is also being replicated. This apparent premature termination is not precipitated by mitotic entry because it also occurs in cycloheximide-arrested extracts where mitosis is prevented (Hutchison et al., 1988, 1989). Once S phase has terminated, addition of either aphidicolin or camptothecin fails to delay mitosis under conditions where addition of either inhibitor at the start of incubation produces considerable mitotic delay (Hynds and Ford, in preparation).

Single-stranded (ss) DNA is an effective template for second-strand synthesis in both egg and oocyte extracts, even when the membrane fraction is removed by high-speed centrifugation (see section Changes between Oocyte and Egg above). This implies that nuclear structure is not necessary for the polymerization reaction. However ss DNA (M13), when added to cycling egg extracts, can delay mitosis in a concentration-dependent manner (Kornbluth et al., 1992). Mitosis is only delayed at ss DNA concentrations (2 μg/μl or greater), which also stimulate detectable second-strand DNA synthesis. Equivalent concentrations of double-stranded (ds) plasmid DNA did not replicate (presumably because this template must be assembled into chromatin and nuclei before replication can be initiated) and did not delay mitosis (Kornbluth et al., 1992). These authors suggest that the process of DNA synthesis stimulates a pathway

that delays mitosis. The ss-DNA template used may be providing a substrate for repair or some aspect of replication sufficient to elicit a signal. Irrespective of which aspect of repair or replication is stimulated, it is sufficient to induce a delay correlated with a high level of tyrosine kinase activity (Kornbluth et al., 1992).

If the process of replication or repair generates a signal that delays mitosis, one might expect the signal to decay once replication or repair is completed. How tyrosine-kinase activity changes following termination of replication or repair remains to be determined. However, an activity that does rise and fall at the expected times of the start and end of S phase in extracts is protein phosphatase I (Walker et al., 1992). This activity also remains elevated in extracts blocked in S phase with aphidicolin. Addition of Inh-2 to an aphidicolin-blocked extract advances mitosis, while addition of protein phosphatase I catalytic subunit delays mitosis in an extract without aphidicolin (Walker et al., 1992). Thus several activities in *Xenopus* extracts appear to be modulated in response to replication arrest or appear to be needed to generate a delay to mitosis when replication is blocked. What is clear is that the presence or absence of nuclei in egg extracts significantly influences the timing of cdc2 kinase activation if those nuclei are capable of undergoing replication. Some component(s) of the nucleus, necessary for the formation of active replication complexes, might signal a delay to mitosis. Loss of such components would then result in a failure to undergo S phase and a failure to prevent mitosis.

V. M PHASE

Fresh egg extracts that cycle between interphase and mitosis have been used to analyze events of mitosis (e.g. Murray et al., 1989), but the precise timing of mitosis can vary and must be determined anew with each extract prepared. Two alternatives have been developed. The first utilizes unfertilized egg (CSF) extracts, which are arrested in meiotic metaphase, with high cdc2 kinase activity and active CSF. Addition of calcium ions (to 0.2–0.4 mM) induces exit into interphase. H1 kinase activity drops substantially, and the cyclin subunit is specifically degraded (Lohka and Maller, 1985; Murray et al., 1989; Lorca et al., 1992).

The second approach has been the development of late interphase or prophase extracts. These extracts are prepared from activated *Xenopus* eggs that are incubated for 60 min before centrifugation. On incubation these extracts display a single cycle of cdc2 kinase activity and cyclin

degradation. As these extracts do not support protein synthesis, they have been used to investigate the posttranslational modifications that regulate both the entry and exit from mitosis (Felix et al., 1989; Felix et al., 1990a,b). While the meiotic and mitotic metaphase states share many similar characteristics, such as high cdc2 kinase activity and the specific degradation of cyclin leading to cdc2 kinase inactivation, the very nature of these cell cycle states suggests that their regulation is different.

A. CSF Extracts

These extracts have been used to study the signals necessary for specific degradation of the cyclins during exit from M phase. The use of modified mRNAs encoding N-terminally truncated forms of sea urchin cyclin B led to the identification of a short amino acid sequence (RAALGNISN) that was necessary for cyclin instability and conferred cell cycle dependent degradation when linked to protein A (Murray et al., 1989; Glotzer et al., 1990). This short sequence is conserved in mitotic cyclins from different species. Extracts treated with truncated cyclin lacking this region were arrested with high cdc2 kinase activity while retaining the capacity to degrade full-length cyclin (Murray et al., 1989). This demonstrated that cyclin degradation was necessary for achieving mitotic exit. Changing the invariant Arg residue in this sequence conferred stability to the protein. It appears that this residue may be required for the poly-ubiquitination that precedes degradation by the ubiquitin pathway (Glotzer et al., 1990).

B. Prophase Extracts

Since synchronous activation of large numbers of eggs can be achieved by electric shock or addition of the Ca^{2+} ionophore A23187, extracts can be prepared at essentially any point in the cell cycle. Once activated, eggs are incubated for the desired length of time before centrifugation. Following a crushing spin at 10,000 ×g for 10 min (4°C) the supernatant is centrifuged for a further 2 hr at 100,000 to 250,000 ×g. The final clear supernatant can be used fresh or snap frozen in liquid nitrogen (Felix et al., 1989).

In vivo the first cleavage usually occurs 90 min after fertilization (at 21°C). The peak of MPF activation for first mitosis occurs at 75 min, and sufficient proteins to achieve this have been synthesized by 45 min (reviewed in Ford, 1985). Prophase extracts prepared 60 min after

activation undergo one cycle of cdc2/H1 kinase activation and inactivation in the absence of protein synthesis, while extracts prepared at 40 min fail to do so, consistent with the protein dependence for mitosis *in vivo* (Felix et al., 1989). Though the precise timing of activation of cdc2 kinase varies slightly from extract to extract, the reproducibility of timing within one extract after freezing is a major advantage.

Both CSF and prophase extracts contain particulate components that are important for extract activity. In both cases, H1 kinase activity and much of the B cyclins are present in the pellet after extended centrifugation, though much of the cdc2 protein remains in the supernanant (Felix et al., 1989; Leiss et al., 1992). Adding NaCl or β-glycerophosphate releases this H1 kinase activity into the final supernatant. There is evidence that cyclin targets cdc2 to an 80S particulate fraction through interphase and that both CSF itself and a component necessary for H1 kinase activation are also particulate (Felix et al., 1989; Shibuya and Masui, 1989; Leiss et al., 1992). Further analysis is required to understand the relationships and roles of these fractions.

C. Calcium and M Phase

The transient increase in intracellular free Ca^{2+} seen after fertilization or activation (Busa and Nuccitelli, 1985) was thought to release the cell from M-phase arrest by triggering the proteolysis of $p39^{c\text{-}mos}$ (Watanabe et al., 1989). Identified as a component of CSF necessary for meiotic arrest (Sagata et al., 1989), $p39^{c\text{-}mos}$ was thought to stabilize MPF by protecting the cyclin subunit from degradation. However it appears that

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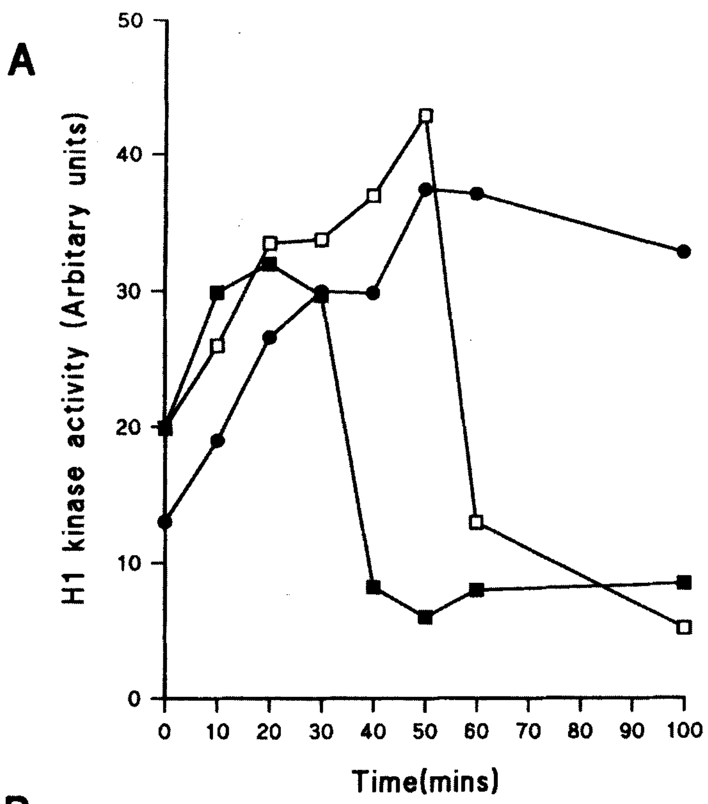
Figure 2. The calcium chelator BAPTA prevents cdc2 kinase inactivation and cyclin degradation in prophase extracts of activated *Xenopus* eggs. A. Extract was incubated at 22°C in the presence of different concentrations of BAPTA; 0 mM (solid squares), 1.5 mM (open squares), 3.0 mM (solid circles). Samples were taken at the times indicated and assayed for cdc2 kinase activity on histone H1 as substrate. B. Cyclin stability was monitored by the addition of ^{35}S labeled *Xenopus* cyclin B_2 translated *in vitro* using a reticulocyte lysate. Samples were diluted in gel sample buffer and run on 12% SDS-PAGE. The gels were then processed for fluorography using Enhance (Dupont). Dried gels were exposed at –80°C using preflashed Hyperfilm-MP (Amersham).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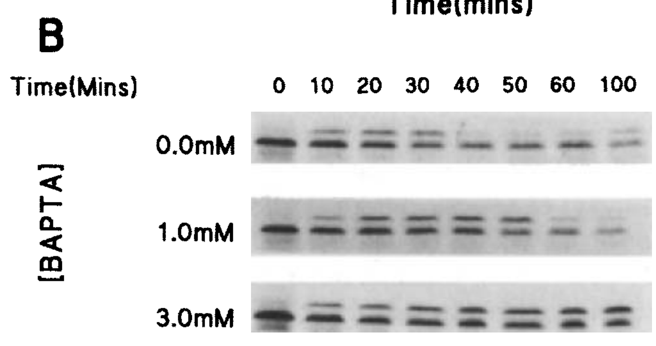
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0.0mM
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MPF inactivation occurs before p39$^{c\text{-}mos}$ is degraded (Lorca et al., 1991b; Watanabe et al., 1991). While the M-phase arrested state may indeed by mediated by p39$^{c\text{-}mos}$, subsequent release is by some other route. This may still be triggered by the Ca^{2+} transient at activation, as cyclin degradation at this point requires Ca^{2+}/calmodulin (Lorca et al., 1991b).

Observations in other systems have pointed to a role for calcium transients in the major rate-limiting steps of mitotic cell cycles (for reviews see Whitaker and Patel, 1990; Hepler, 1989; Anraku et al., 1991). In *Xenopus* eggs injection of EGTA/Ca^{2+} mixes has been shown to inhibit cleavage, though it was unclear whether eggs were arrested due to a block in cytoplasmic cleavage or whether some other events such as nuclear division were being affected (Baker and Warner, 1972). The observation of oscillations of intracellular free calcium in phase with the cell cycle has provided further evidence for the involvement of calcium in the *Xenopus* cell cycle (Grandin and Charbonneau, 1991). However, prophase extracts are prepared with a buffer containing 60 mM EGTA, generating a concentration of some 20 mM in the final extract (Felix et al., 1989). Despite EGTA at this concentration, these extracts efficiently degrade both exogenous and endogenous cyclin (Felix et al., 1989, Lindsay and Ford, unpublished). This would argue against a Ca^{2+} requirement for cyclin degradation at mitosis exit. However, levels of free Ca^{2+} in these extracts are in the range 200 to 500 nM (Lindsay et al., 1995). These levels are consistent with those found both in eggs (Grandin and Charbonneau, 1991, 1992) and in CSF extracts (Lorca et al., 1991b).

We have investigated the effect of calcium chelators on the activation and deactivation of cdc2 kinase in 250,000 ×g prophase extracts to see whether calcium regulates any of the posttranslational modifications required for cdc2 kinase activity in some way. We chose BAPTA (1,2-bis(2-aminophenoxy)-ethane-N,N,N′-tetraacetic acid) in preference to EGTA as it has a higher affinity for calcium and is largely unaffected by changes in pH (Tsien, 1980). Addition of BAPTA to prophase extracts similar to those characterized by Felix et al. (1989), allows the spontaneous activation of H1 kinase activity but prevents deactivation and cyclin degradation (Figure 2). Extracts arrest with high levels of H1 kinase activity and stable cyclin. Addition of calcium buffered BAPTA at the same concentration (Figure 3) allowed kinase activation and deactivation, indicating that BAPTA is influencing events through its calcium chelating properties (Lindsay et al., 1995). These observations

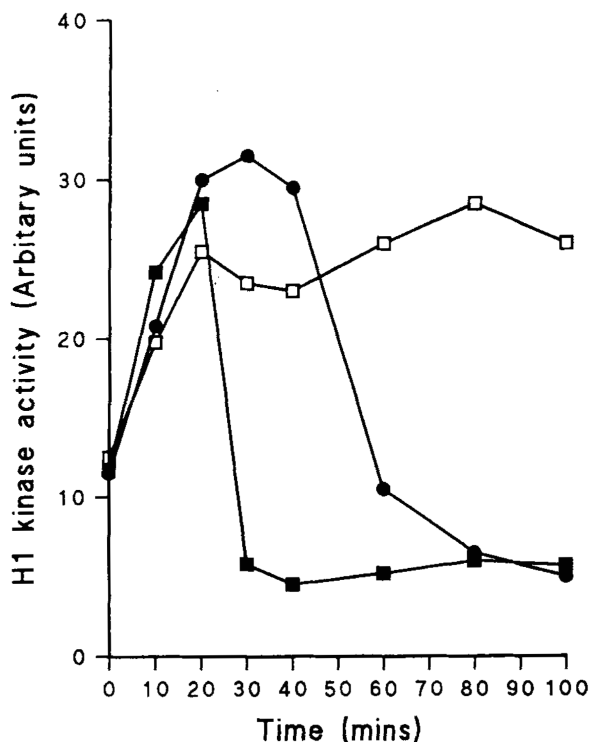

Figure 3. Preincubation of BAPTA with calcium rescues cdc2 kinase deactivation. Extract was incubated at 22°C in the presence of a concentration of BAPTA that would be expected to prevent kinase deactivation (3 mM). BAPTA was premixed with calcium before addition to extract. Water control (solid squares), 3 mM BAPTA + water (open squares), 3 mM BAPTA + 1.5 mM Ca^{++} (final concentrations, solid circles). Samples were taken at the times indicated and assayed for cdc2 kinase activity. The timing of cdc2 kinase deactivation is slightly delayed in relation to the control. This appeared to be a direct result of the BAPTA/$CaCl_2$ ratio used for a particular condition, as the timing of deactivation could be altered by small increases or decreases in the concentration of Ca^{2+} mixed with the constant concentration of BAPTA.

suggest that a calcium mediated event is required for cdc2 kinase inactivation and, therefore, for mitotic exit in *Xenopus* extracts.

In sea urchin eggs there is evidence that calcium-mediated events influence both mitotic entry and exit. Injection of BAPTA into fertilized *L. pictus* eggs results in the arrest of the cell cycle in G_2 (Twigg et al., 1988). Cell cycle progression can then be rescued by the subsequent injection of calcium (Twigg et al., 1988; Patel et al., 1989). Injection of BAPTA into eggs during mitosis results in mitotic metaphase arrest (Whitaker and Patel, 1990).

As yet, the targets for these Ca^{2+} mediated events are unknown; but for them to be able to affect cell cycle events, it is likely that they integrate into the pathways that affect the activation and inactivation of cdc2 kinase. It is therefore probable that the calcium-dependent phosphatases (type 2B) and/or kinases are involved. The injection of antibodies or inhibitory peptides directed at a type II calmodulin-regulated kinase arrests cell cycle progression in G_2 in sea urchins (Baitinger et al., 1990). However the expression of a constitutively active form of CAM kinase II in a mammalian cell line also results in arrest in G_2 (Planas-Silva and Means, 1992). These results suggest that CaM kinase may influence mitotic entry in different ways depending on the particular cell type under consideration.

Normally release from meiotic metaphase arrest involves a calcium transient. However the calcium requirement can be bypassed in certain circumstances. Using CSF extracts, Lorca et al. (1991a) have shown that without calcium addition, cyclin degradation and cdc2 kinase inactivation can be induced by addition of the phosphatase inhibitor, okadaic acid. Unlike the Ca^{2+}-mediated meiotic exit, okadaic acid treated CSF extracts retain CSF activity, suggesting that okadaic acid is bypassing the regulatory system mediated by CSF (Lorca et al., 1991a). This also suggests that CSF normally maintains the activity of a phosphatase (probably phosphatase 2A). Calcium-mediated inactivation of CSF would then lead to a reduction in phosphatase 2A activity and activation of cyclin degradation. If, however, the calcium transient at fertilization leads independently to inactivation of CSF and protein phosphatase 2A, then both meiotic and mitotic exit would require a calcium mediated signal to instigate cyclin degradation.

VI. CONCLUSION

The concentrated cytoplasmic extracts derived from *Xenopus* early embryonic material are exceptional in the range of complex cellular activities they carry out in a manner that is seductively similar to events *in vivo*. However, occasionally the behavior can be strikingly dissimilar. For instance, in extracts that cycle multiple times, spindles do not form during the mitotic phases. Despite this, it is quite clear that these extracts provide not only a rich source of molecules involved in a wider range of activities than we have considered but also a readily manipulable system in which to probe aspects of the structural and biochemical events that keep the egg's clock ticking.

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VII. NOTE ADDED IN PROOF

Fortunately since this review was written, the behavior of extracts from *Xenopus* oocytes and eggs, and the methods used in their preparation have not changed though insight into the events that they support has.

Initiating S phase requires replication protein A, which associates rapidly in a punctate pattern with decondensing chromatin (Adachi and Laemmli, 1994). Members of the MCM family of proteins are also required for S phase initiation, associate rapidly with chromatin, but dissociate again during S phase (Kubota et al., 1995; Chong et al., 1995; Madine et al., 1995). Initiation of replication is inhibited by a phosphatase 2A inhibitor (Murphy et al., 1994) and by Cip 1, a member of the group of cdk inhibitors (Strausfeld et al., 1994; Yan and Newport, 1995). S phase regulation has been reviewed recently (Coverley and Laskey, 1994; Chevalier et al., 1995; see section IV B).

The RCC1 protein, required for replication and preventing premature chromosome condensation interacts with Ran/TC4 and several other proteins (Saitoh and Dasso, 1995). Ran/TC4 protein influences nuclear structure and progression to mitosis (Dasso et al., 1994; Kornbluth et al., 1994; Clarke et al., 1995). Evidence in yeast links DNA polymerase ε in the replication machinery with the S phase checkpoint (Navas et al., 1995, see section IV C).

The role of calcium in the cell cycle has been reviewed recently (Lu and Means, 1993). In *Xenopus* extracts inositol trisphosphate induced calcium release appears to be necessary for membrane fusion during nuclear assembly (Sullivan et al., 1994, see section III B). Calcium/calmodulin-dependent protein kinase II is involved in the onset of anaphase and specific cyclin proteolysis during release of CSF extracts into interphase (Lorca et al., 1993; Morin et al., 1994). Homologues of yeast CDC27 and CDC16 proteins form part of a 20S complex in egg extracts that catalyses specific cyclin ubiquitination during mitosis leading to cyclin degradation (King et al., 1995; see section V A, C).

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