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Xenopus Oocytes and the Biochemistry of Cell Division†

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ABSTRACT: The control of cell proliferation involves both regulatory events initiated at the plasma membrane that control reentry into the cell cycle and intracellular biochemical changes that direct the process of cell division itself. Both of these aspects of cell growth control can be studied in *Xenopus* oocytes undergoing meiotic maturation in response to mitogenic stimulation. All mitogenic signaling pathways so far identified lead to the phosphorylation of ribosomal protein S6 on serine residues, and the biochemistry of this event has been investigated. Insulin and other mitogens activate ribosomal protein S6 kinase II, which has been cloned and sequenced in oocytes and other cells. This enzyme is activated by phosphorylation on serine and threonine residues by an insulin-stimulated protein kinase known as MAP-2 kinase. MAP kinase itself is also activated by direct phosphorylation on threonine and tyrosine residues in vivo. These results reconstitute one step of the insulin signaling pathway evident shortly after insulin receptor binding at the membrane. Several hours after mitogenic stimulation, a cell cycle cytoplasmic control element is activated that is sufficient to cause entry into M phase. This control element, known as maturation-promoting factor or MPF, has been purified to near homogeneity and shown to consist of a complex between $p34^{cdc2}$ protein kinase and cyclin B2. In addition to apparent phosphorylation of cyclin, regulation of MPF activity involves synthesis of the cyclin subunit and its periodic degradation at the metaphase → anaphase transition. The $p34^{cdc2}$ kinase subunit is regulated by phosphorylation/dephosphorylation on threonine and tyrosine residues, being inactive when phosphorylated and active when dephosphorylated. Analysis of phosphorylation sites in histone H1 for $p34^{cdc2}$ has revealed a consensus sequence of $(^{K}/_{R})^{S}/_{T}P(X)^{K}/_{R}$, where the elements in parentheses are present in some but not all sites. Sites with such a consensus are specifically phosphorylated in mitosis and by MPF in the protooncogene $pp60^{c\text{-}src}$. These results provide a link between cell cycle control and cell growth control and suggest that changes in cell adhesion and the cytoskeleton in mitosis may be regulated indirectly by MPF via protooncogene activation. S6 kinase II is also activated upon expression of MPF in cells, indicating that MPF is upstream of S6 kinase on the mitogenic signaling pathway. Further study both of the signaling events that lead to MPF activation and of the substrates for phosphorylation by MPF should lead to a comprehensive understanding of the biochemistry of cell division.

Cell proliferation as a process encompasses a vast array of biological phenomena. Included in the process are the transduction of extracellular signals into intracellular biochemical signals, the complex structural reorganization of the cellular architecture to prepare for a division cycle, and finally the reprogramming of metabolism to actually carry out cell division. Each of these aspects usually is studied as an isolated process with a particular cell type or model system. However, it seems clear that in order to gain a comprehensive understanding of cell proliferation, it will be necessary to study all of these phenomena as an integrated process in a single cell type. The purpose of this paper is to review the progress that has been made in each of these areas in the *Xenopus* oocyte system and to propose that the oocyte is uniquely suited to provide an understanding of how extracellular signals are transduced into intracellular biochemical signals that activate regulatory elements of cell cycle control, which reorganize cellular metabolism for division. The central process that underlies all aspects of cell proliferation analysis is the cell cycle. Therefore, in the first part of this paper, I will review briefly the general concept of cell cycle control that has emerged from studies in somatic cells and then consider the

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oocyte system in relation to this general picture.

Cell Cycle Restriction Points. Two general restriction points have been identified in the cell cycle, one in the G_0/G_1 period governing entry into S phase and the other in the G_2 phase governing entry into mitosis [for reviews, see Pardee et al. (1978), Marcus et al. (1985), and Baserga (1981)]. Since both DNA synthesis and mitosis are hallmarks of cellular reproduction, analysis of both of these restriction points is valid for cellular control processes. Although the G_0 state is believed to exist in vivo in various types of stem cells, experimentally it has been defined in relation to in vitro culture of somatic cells. Generally, cultured mammalian cells require the addition of external growth factors, present in serum, in order to undergo a round of DNA synthesis and cell division. By removal of serum leading to cell cycle arrest in a G_0 state, the events that accompany reentry into the cell cycle can be studied upon readdition of defined growth factors. The particular growth factors required appear to depend on the cell type and the exact stage of G_0 arrest. In some cases growth factors make cells competent to reenter the cell cycle upon treatment with other factors, while in other cases cell cycle progression is directly stimulated. Analysis of growth factor action has been carried out at several levels. The receptors to which growth factors bind have been studied intensively, and the most progress has been made with receptors that express a tyrosine protein kinase activity, as in the case of EGF, PDGF, insulin, and IGF_1 receptors [see Yarden and Ullrich (1988) for a review]. Binding of these growth factors has several immediate consequences. The first is activation of the tyrosine protein kinase activity of the receptor itself, which has been shown in most cases to correlate with the appearance of new phosphotyrosyl proteins. In several cases, this phosphorylation is closely correlated with changes in phospholipid metabolism. Within the next few minutes, several immediate early events occur that appear to be of general significance. One is the transcription of several genes, notably c-*fos*, c-*myc*, and c-*jun*, whose protein products are transcription factors that probably activate as yet unidentified genes for events later in the cell cycle. At the biochemical level, two immediate early events are seen in most cell types. One is an increase in intracellular pH due to Na/H exchange, and the other is the phosphorylation on serine residues of ribosomal protein S6. Although both these events are very highly correlated with mitogenic stimuli, the exact function of the pH increase or of S6 phosphorylation has not been established. In the case of S6 phosphorylation, it has not yet been possible to artificially increase or block S6 phosphorylation, which would be necessary to evaluate its potential importance. However, it seems unlikely that S6 phosphorylation itself is sufficient to increase the rate of protein synthesis. In spite of the uncertain regulatory significance of these events, the high conservation of their expression encourages analysis of their regulation as models for the biochemistry of early mitogenic activation, especially by stimuli that work through tyrosine kinase activation.

It is evident from the analysis of regulation at the G_0/G_1 transition that cell cycle progression is characterized by both transcriptional and translational regulation and is most likely concerned with the immediate reprogramming of the cellular metabolism for DNA synthesis. It is probable that different changes are needed for cells to pass through the G_2 restriction point.

G_2 Restriction Point. Like the G_0/G_1 restriction point, it appears that in vivo some cell populations are physiologically arrested in G_2 phase (Pedersen & Gelfant, 1970; Melchers & Lernhardt, 1985). A number of cultured cells can be artificially blocked in G_2 by elevating the level of cAMP (Nose & Katsuta, 1975; Stambrook & Velez, 1976), suggesting that protein kinase A exerts an inhibitory effect on cell division at this point in the cell cycle. Biochemical support for a G_2 block has been particularly convincing from studies on various types of oocytes, where a physiological G_2 block is released by a reduction in cAMP levels (Maller et al., 1977; Meijer & Zarutskie, 1987; Bornslaeger et al., 1986).

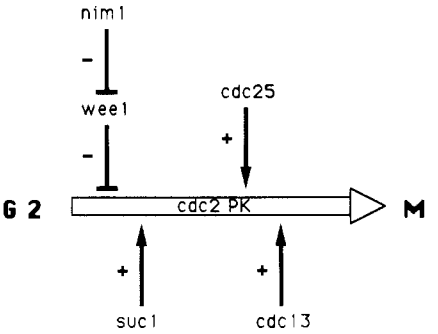

FIGURE 1: Genetic network regulating the eucaryotic cell cycle. The figure depicts the *cdc2*$^+$ gene prodct as the central regulator of the $G_2 \rightarrow M$ transition. Other genes indicated are known to be required either for activating *cdc2* kinase or for specifying the timing of *cdc2* kinase activation in the cell cycle, as described in the text. Modified from Russell and Nurse (1987b).

In both the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, elegant genetic analysis has shown that mutations in several genes lead to an arrest at the G_2/M border of the cell cycle [see Lee and Nurse (1988) for a review]. The analysis has been more extensive in *S. pombe* because it has a cell cycle more like that of vertebrate cells, with well-defined G_1 and G_2 periods. Figure 1 shows a scheme proposed by Nurse's laboratory that takes into account the genetic analysis to date (Russell & Nurse, 1987b). The central control element regulating the $G_2 \rightarrow M$ transition is cell division cycle gene 2 (*cdc2*$^+$). This gene encodes a 34-kDa serine/threonine protein kinase. While $p34^{cdc2}$ has all the usual consensus sequences for protein kinases, the remaining sequence is not closely related to any other member of the protein kinase family that has been sequenced.

Mutations in *cdc2*$^+$ that cause arrest in G_2 have been intensely studied, although in some cases mutants arrest in G_1, indicating that this protein kinase is involved in both G_1/S and G_2/M control points in the cell cycle [for reviews, see Nurse (1985) and Lee and Nurse (1988)]. Suppressors of mutations in *cdc2*$^+$ have fallen into three classes. About half of the suppressors involve a gene known as *sucI*, which encodes a protein that binds to $p34^{cdc2}$ in vitro and stimulates the kinase activity in extracts of thermolabile mutants (Brizuela et al., 1987; Moreno et al., 1989; Dunphy et al., 1988). The exact function of the *sucI* gene is unknown at present, but deletion of *sucI* leads to mitotic arrest, suggesting a role in exit from mitosis (Moreno et al., 1989). This role is likely to be important inasmuch as *sucI* homologues have been identified in human cells (Draetta et al., 1987). Over- or underexpression of *sucI* does not appear to affect the timing of mitosis.

A positive stimulus regulating the timing of *cdc2*$^+$ activation is evident after expression of the *cdc25* gene, whose sequence is unrelated to any other sequence in the gene bank. Other timing genes include *wee1*$^+$ and *nim1*$^+$, both of which encode proteins with consensus sequences for protein kinases (Russell & Nurse, 1987a,b). *wee1*$^+$ inhibits the timing of *cdc2*$^+$ activation, while *nim1*$^+$ stimulates *cdc2*$^+$ by a mechanism that involves *wee1*$^+$. The timing controls on *cdc2*$^+$ by *cdc25* and

wee1$^+$ are independent, but they can be affected by mutations in other genes that encode homologues of protein phosphatase 1 (Booher & Beach, 1989). Fission yeast *wee1*$^+$ has been shown to exert mitotic control when expressed in the distantly related budding yeast, *S. cerevisiae*, and its action is opposed by the *MIH1* gene, a budding yeast homologue of *cdc25*$^+$ (Russell et al., 1989). Another element required for *cdc2* kinase activation is the product of the *cdc13*$^+$ gene, which is required for activation of *cdc2* in mitosis and may also be necessary for spindle microtubule organization (Moreno et al., 1989; Hagan et al., 1988; Booher & Beach, 1988). Sequence analysis indicates the *cdc13*$^+$ gene product is similar to the cyclin proteins implicated in mitotic control in the eggs of various marine invertebrates (Evans et al., 1983; Solomon et al., 1988; Goebl & Byers, 1988). These results indicate that the *cdc2*$^+$ gene product is regulated by a complex network of other proteins, including other protein kinases, and suggest that protein phosphorylation will prove to be the fundamental biochemical process underlying the molecular basis of the $G_2 \rightarrow M$ transition in the cell cycle.

The function of *cdc2*$^+$, and presumably its kinase specificity, has been highly conserved in evolution. *cdc2*$^+$ is a homologue of a gene in budding yeast known as *CDC28*$^+$, which has been cloned and sequenced and found to also encode a 34-kDa serine/threonine protein kinase. Each homologue is able to complement mutations in the other gene when expressed in the heterologous yeast (Beach et al., 1982). More specifically, the human homologue of *cdc2*$^+$ was also cloned by complementation (Lee & Nurse, 1987), indicating that the function of this gene in cell cycle control is conserved from yeast to man. Despite the clear importance of *cdc2*$^+$ and the delineation of a genetic regulatory network for the $G_2 \rightarrow M$ transition, little biochemical information has been forthcoming from this system. Instead, most biochemical information on the $G_2 \rightarrow M$ transition has come from the *Xenopus* oocyte system, as discussed in the next section.

Xenopus Oocyte System. Like most vertebrates, oocytes from *Xenopus* are physiologically arrested at the G_2/M border in first meiotic prophase. In amphibians like *Xenopus*, release from this prophase block occurs in response to mitogenic stimulation by progesterone, insulin, or IGF_1. Several hours later entry into meiosis I is signaled by breakdown of the germinal vesicle (nucleus), termed GVBD. Because of the perfect synchrony of the oocyte population prior to mitogenic stimulation, biochemical changes are more easily monitored even if they have transient kinetics. Many of the changes seen in oocytes undergoing maturation are similar to those described for cultured cells traversing the G_0/G_1 restriction point after growth factor addition. For analysis of phosphorylation events involved in the $G_2 \rightarrow M$ transition, we have focused on the activation of ribosomal protein S6 as a defined biochemical end point in the signaling pathway used by all mitogens so far studied, with the objective of working backward up a pathway, ultimately reaching the mitogen receptor itself. The particular mitogen we have chosen to focus on is insulin, in part because the question of signaling by tyrosine kinases is a major unresolved problem in cell growth and cell cycle control. One reason for working backward up the pathway is the disappointing lack of progress in any cell type in characterizing initial substrates or initial steps in the insulin signaling pathway beyond activation of the tyrosine kinase activity of the insulin receptor. Oocytes have genuine insulin receptors as judged by insulin binding, anti-insulin receptor antibody binding, and inhibition of insulin effects by microinjected antibodies against the tyrosine kinase domain of the β subunit (Maller & Koontz,

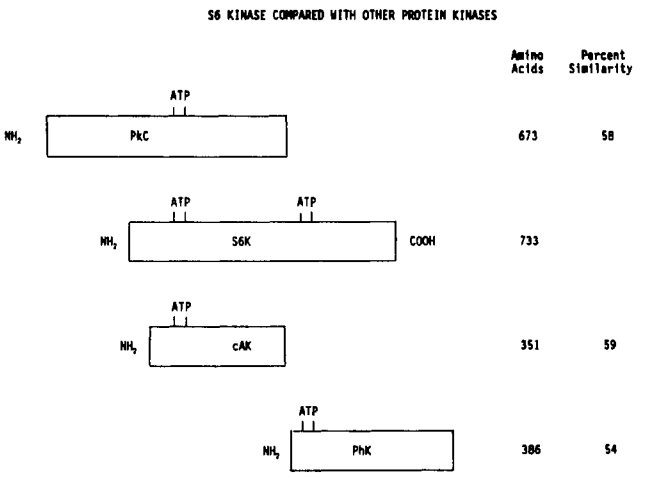

FIGURE 2: Sequence similarity of kinase domains in S6KII. The figure shows schematically that S6KII contains two apparent consensus sequences for ATP-binding sites in protein kinases, one in each half of the molecule. The kinase domain in the amino-terminal half is 58% similar at the amino acid level to protein kinases A and C, while the domain in the C-terminal half is more similar to phosphorylase *b* kinase. Whether both domains are catalytically active is unknown at present.

1980; Morgan et al., 1986). However, oocytes also possess structurally similar IGF_1 receptors, and it is possible all of these criteria actually measured IGF_1 receptors. Because of the very close structural and functional relationship between insulin and IGF_1 receptors, it is likely that information about signaling pathways for either receptor will have general significance.

Upon stimulation of oocytes with insulin, there is a rapid activation of an S6 kinase activity within 15–30 min that returns to near basal by 1 h post insulin (Stefanovic et al., 1986; Stefanovic & Maller, 1988). From 2 to 3 h after insulin, S6 kinase activity begins to increase again, reaching a maximal rate just prior to GVBD at 6–7 h post insulin. This maximal rate of increase in S6 kinase activity correlates with a large 3–5-fold increase in protein phosphorylation that has been called the "burst" of phosphorylation (Maller et al., 1977; Doree et al., 1983). This event is a hallmark of biochemical reprogramming of the cell for division and is evident about 1 h prior to nuclear breakdown. The early increase in S6 kinase activation is unaffected by treatment of oocytes with cycloheximide, while the burst of phosphorylation is blocked by pretreatment with cycloheximide (Stefanovic & Maller, 1988). Because S6 kinase activation is maximal at the completion of maturation, metaphase II arrested unfertilized eggs, which have undergone maturation in vivo, were used as a source of material for purification. DEAE-Sephacel profiles of S6 kinase activity in eggs revealed two peaks of non-cAMP-dependent activity, termed S6KI and S6KII in order of their elution from the column. Purification of S6KII to homogeneity yielded a single polypeptide chain of M_r 92000 on Laemmli gels, while S6KI is an M_r 90000 protein (Erikson & Maller, 1986, 1989a). Both kinases are relatively specific for S6 although S6KI can also weakly phosphorylate histone H1 or α-casein, common substrates for broad specificity kinases. One hundred micrograms of S6KII was purified from 1.2 kg of eggs, tryptic peptides were sequenced, and oligonucleotide probes were constructed for screening of a *Xenopus* ovary cDNA library in λgt10. Two clones that were sequenced showed 96% identity with each other, and antibodies against the bacterially expressed protein reacted with purified S6KII (Jones et al., 1988). The sequence of S6KII proved to be rather interesting, since there were apparently complete consensus sequences for protein kinase domains in both the N-

terminal and C-terminal halves of the molecule (Figure 2). The N-terminal domain is 58% similar in sequence to the sequences of protein kinases A and C, while the C-terminal half is much more similar to the catalytic domain of phosphorylase *b* kinase. Whether both catalytic domains are active has not yet been established. However, the presence of two domains encouraged additional examination of the substrate specificity of S6KII, and it was found to also phosphorylate glycogen synthase, tyrosine hydroxylase, troponin I, and lamin C (Erikson & Maller, 1988). These phosphorylation sites were found to exhibit a consensus motif of RXXS. The phosphorylation of lamin C occurs at a site increased in mitosis when disassembly of the nuclear envelope occurs, suggesting that S6KII may be directly involved in disassembly of the envelope at GVBD (Ward & Kirschner, 1988).

Recent data indicate that S6 kinases of similar size and sequence are present in mouse and chicken cells, react with antibodies against bacterially expressed S6KII, and are activated in response to mitogenic stimulation (Alcorta et al., 1989). In cells transformed by a temperature-sensitive mutant of $pp60^{v\text{-}src}$, an S6KII homologue is activated in a temperature-sensitive fashion, suggesting that S6KII is a target for oncogene action (Erikson et al., 1987). These results suggest S6KII and its homologues are a family of highly conserved kinases involved in mitogenic stimulation. While S6KII is clearly a major partner in the mechanism of mitogenic stimulation of S6 phosphorylation, these data do not exclude that other S6 kinases are also activated by mitogens. Enzymes of lower molecular weight have been described in frogs (S6KI), chicken (Blenis et al., 1987), mouse (Jeno et al., 1989), and rat (Price et al., 1989) that may be members of a distinct group of S6 kinases, possibly more involved in secondary waves of S6 kinase activation after initial mitogen treatment.

Interest in this laboratory is focused on the pathway of activation of S6 kinase rather than on detailed molecular characterization of the enzyme. Thus we were excited by the initial finding that incubation of S6 kinase II with either protein phosphatase 1 or 2A caused up to a 98% inactivation of the enzymatic activity (Maller, 1987; Sturgill et al., 1988). Since these two phosphatases are major serine/threonine-specific phosphatases in cells (Ingebritsen & Cohen, 1983), it suggested that the next step back in the signaling pathway was another serine/threonine protein kinase. It was reported that Swiss 3T3 cell S6 kinase could also be inactivated by phosphatase (Ballou et al., 1988), suggesting that S6 kinases in general are activated by direct phosphorylation rather than be second messengers. A screening of a variety of broad specificity kinases, such as protein kinases A and C, demonstrated no reactivation of S6 kinase activity under phosphorylation conditions. However, a purified, highly specific, insulin-stimulated protein kinase from 3T3-L1 cells able to phosphorylate microtubule-associated protein 2 (MAP-2) phosphorylated and reactivated dephospho-S6KII (Sturgill et al., 1988). Phosphopeptide mapping demonstrated that two new phosphopeptides were present in S6KII after MAP kinase phosphorylation and, significantly, reactivation was accompanied by the de novo appearance of phosphothreonine in S6KII. Recently S6KII has also been shown to be phosphorylated in vivo on serine and threonine residues as it becomes activated during maturation induced by a variety of mitogens (Erikson & Maller, 1989a). The phosphopeptide map of in vivo activated S6KII is more complex than the in vitro pattern for S6KII activated with MAP kinase induced by a variety of stimuli. This is not unexpected, since phosphatase 2A causes 98% inactivation of S6KII and MAP kinase can only restore

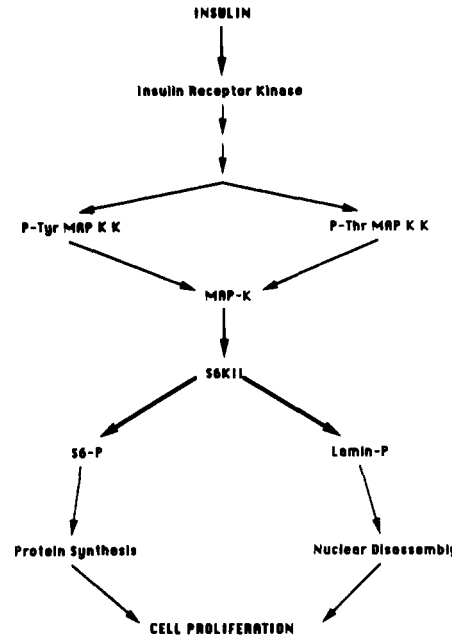

FIGURE 3: Possible signaling pathway for activation of S6KII. The diagram shows S6KII activation by MAP kinase, which is itself activated by two phosphorylation events mediated by different kinases, one tyrosine and one serine/threonine. Several additional intermediates are likely to exist between the MAP kinase kinases and the insulin receptor itself. These results suggest two types of signaling pathways are required for activation of MAP kinase and thus S6KII.

30% of the activity, implying the existence of other S6KII kinases able to phosphorylate other sites in S6KII evident in in vivo mapping. Activation of S6 kinase by phosphorylation is correlated with a decrease in electrophoretic mobility on SDS gels, permitting Western blotting to be used for assessing phosphorylation and dephosphorylation. Analysis by this method has shown that all S6 kinase molecules become phosphorylated during maturation and all become dephosphorylated upon egg activation (Erikson & Maller, 1989a).

These results demonstrate the reconstitution of one step in the insulin signaling pathway for the $G_2 \rightarrow M$ transition and support the concept that working backward up the signaling pathway is a productive approach. Although the concept of kinase cascades dominates the literature of protein phosphorylation, this S6KII system is only the third example of one serine/threonine kinase phosphorylating and activating another serine/threonine kinase, the classic case being the phosphorylation of phosphorylase kinase by cAMP-dependent protein kinase (Walsh et al., 1968). Ultimately, these results lead us to consider the activation of MAP kinase as the next step back in the signaling pathway. Since MAP kinase was known to be phosphorylated on threonine and tyrosine residues in vivo after insulin stimulation (Sturgill & Ray, 1988), MAP kinase was incubated with phosphatases 1 and 2A and the activity of MAP kinase assessed. Phosphatase 1 had no effect on MAP kinase, but phosphatase 2A caused over an 80% inactivation of MAP kinase activity (Sturgill et al., 1988). Phosphoamino acid analysis of dephosphorylated MAP kinase confirmed that inactivation correlated with removal of phosphothreonine (T. Sturgill, personal communication). Although the function of tyrosine phosphorylation of MAP kinase is unknown at present, it is clearly not sufficient to activate the enzyme, and attempts to phosphorylate MAP kinase in vitro with purified insulin receptor kinase have so far been unsuccessful. However, these results show that MAP kinase is also regulated by direct phosphorylation and point to the existence of a serine/threonine MAP kinase kinase as being important in the signaling

pathway. The pathway depicted in Figure 3 summarizes this discussion. It should be emphasized that a large number of steps are likely to be present between activation of the insulin receptor tyrosine kinase activity and activation of MAP kinase. In addition, as mentioned earlier, other mitogenic stimuli also cause activation of oocyte S6KII by phosphorylation. It is not yet clear whether these other stimuli represent completely independent but parallel stimuli or whether they intersect at MAP kinase or at a point further upstream in the insulin signaling pathway.

Analysis of Maturation-Promoting Factor. As mentioned earlier, S6 phosphorylation is a small component of the burst of phosphorylation observed to occur in oocytes 30–60 min prior to GVBD. This burst of phosphorylation represents a pleiotropic response to activation of maturation-promoting factor (MPF). Thus in working backward up the late pathway of S6 kinase activation, it could be predicted that MPF will be an intermediate between the receptor and MAP kinase kinase. MPF was first identified by cytoplasmic transfer experiments in which metaphase cytoplasm induced precocious maturation when injected into resting oocytes in the absence of mitogenic factors or protein synthesis (Masui & Markert, 1971; Smith & Ecker, 1971). Subsequently, an activity with similar properties able to induce GVBD in *Xenopus* was described in oocytes of other species and in mitotic cells from yeast to man, suggesting MPF was likely to be of fundamental importance in cell division and might have evolutionarily conserved components. Little was known about the molecular nature of MPF from studies using the microinjection assay. However, it was shown that oocytes contain a latent store of MPF, since a large (>100-fold) amplification of MPF activity was evident within 2 h in injected oocytes, even in the presence of protein synthesis inhibitors (Reynhout & Smith, 1974; Gerhart et al., 1984; Cyert & Kirschner, 1988). Correlated with the amplification was the large burst of phosphorylation also evident just prior to GVBD in mitogen-stimulated oocytes undergoing maturation (Maller et al., 1977). This led to the hypothesis that MPF was a kinase or an activator of a kinase. Other work using the oocyte injection assay showed that MPF was already present in latent form in small oocytes unable to respond to external mitogenic administration, suggesting that the activation mechanism for MPF was under acute developmental control (Hanocq-Quertier et al., 1976; Sadler & Maller, 1983; Wasserman et al., 1984; Taylor & Smith, 1985). Wasserman and Smith (1978) were the first to show that MPF activity oscillated during the mitotic cell cycle after fertilization, and Gerhart et al. (1984) showed that MPF activity also oscillated between meiosis 1 and 2 in *Xenopus*. Oscillations in the total level of phosphorylation were also found to occur in various species in concert with changes in MPF activity (Doree et al., 1983; Peaucellier et al., 1984).

For nearly 15 years, little progress was made in MPF purification using the oocyte injection assay, although several attempts were reported (Wu & Gerhart, 1980; Adlakha et al., 1985; Nguyen-Gia et al., 1986). In retrospect, two main reasons for difficulty are evident. One is the instability of MPF, and the other is the requirement for activity to be highly concentrated in order to give GVBD in oocytes. Additionally, since the oocyte only has one nucleus, the end point is all or none. This situation changed because of two key developments—improved stabilization procedures and a cell-free assay for MPF. Extraction of MPF was markedly improved by the inclusion of EGTA and β-glycerophosphate in the medium, and stability was further enhanced by the inclusion of γ-S-ATP in the extraction. Since thiophosphorylated proteins are often poor substrates for protein phosphatases, this suggested that maintenance of a phosphoprotein was important in stabilizing MPF activity.

Xenopus Cell-Free System. The most significant development in the characterization of MPF was the generation by Manfred Lohka of a cell-free system from unfertilized, metaphase-arrested frog eggs that could carry out early mitotic events in vitro in response to MPF (Lohka & Masui, 1984; Lohka & Maller, 1985; Miake-Lye & Kirschner, 1985). These events include nuclear envelope breakdown, chromosome condensation, and spindle formation. If eggs are in interphase at the time of extraction, the preparation induces nuclear assembly and total semiconservative DNA replication instead of mitotic events (Blow & Laskey, 1986). Interconversion from M phase to interphase could be accomplished by addition of calcium, and conversion from interphase to M phase could be accomplished by addition of MPF even in the presence of protein synthesis inhibitors (Lohka & Maller, 1985). The development of a cell-free system able to carrry out a single cell cycle is a breakthrough in the biochemistry of cell division, and the system has been rapidly adopted by a number of workers. For the first time, the control mechanisms regulating entry into and exit out of M phase can be studied functionally in vitro, and the individual component processes of mitosis such as nuclear breakdown, chromosome condensation, and spindle formation are now able to be studied in a functional, reversible in vitro system. This cell-free system promises to have a profound impact on cell biology. Already, Murray and Kirschner have shown that if much more concentrated interphase egg extracts are prepared, multiple cell cycles of alternating M phase and DNA synthesis occur in vitro spontaneously, permitting study of the entire cell cycle in a dynamic in vitro system (Murray & Kirschner, 1989; Murray et al., 1989).

As a first use of the MPF-dependent single cell cycle system, Lohka et al. (1988) purified MPF 3500-fold to near homogeneity, using as an assay breakdown of synthetic nuclei in the cell-free system. Purified MPF was also able to induce GVBD when injected into oocytes in the presence of cycloheximide. The purified preparation consisted largely of two polypeptide chains of M_r 32 000–34 000 and 45 000. Upon incubation with [γ-^{32}P]ATP, the 45-kDa component became phosphorylated on serine and threonine residues, but only in fractions that also contained the 34-kDa component. This indicated MPF was a complex of a 34-kDa serine/threonine protein kinase and a 45-kDa subunit.

Identification of p34^{cdc2} in MPF. The finding that MPF contained a 34-kDa protein kinase was striking because of the extensive genetic work described earlier in the fission yeast *S. pombe* that had identified the *cdc2*$^+$ gene as a central regulator of the $G_2 \rightarrow M$ transition in the cell cycle; the *cdc2*$^+$ gene encodes a 34-kDa serine/threonine protein kinase.

Work by Nurse and his colleagues has shown that while there is only 63% sequence identity between the functionally interchangeable human and fission yeast *cdc2* products, all *cdc2* homologues show perfect conservation of a 16 amino acid PSTAIR region unrelated to any normal kinase consensus sequence (Lee & Nurse, 1987, 1988). Antibodies raised against a synthetic peptide corresponding to this sequence detected a single protein in Western blots of extracts of a wide variety of cells, which suggested they were specific for p34^{cdc2}. The PSTAIR sequence itself is likely to be a functionally important domain for *cdc2*, inasmuch as a synthetic peptide encoding the sequence has biological effects in the cell-free system or in injected oocytes (Gautier et al., 1988; Labbe et

al., 1989a). However, recently other genes were reported to contain sequences containing 14 of the 16 amino acids, indicating that some caution must be exercised in the use of this antibody (Toh-e et al., 1988). However, this antibody was able to detect by Western blot and to immunoprecipitate the 34-kDa component of MPF, providing direct evidence that $p34^{cdc2}$ was the kinase component of MPF (Gautier et al., 1988). Indirect evidence supporting the same conclusion was obtained at the same time by Dunphy et al. (1988), who showed that the product of the *sucI* gene, known as p13, which binds the product of *cdc2*, could inhibit MPF activity in the cell-free system and deplete MPF activity from extracts. A large number of other proteins also bound to $p13^{sucI}$–Sepharose beads in extracts, including a 42-kDa protein (Dunphy et al., 1988) that is unrelated to the p45 component seen in purified MPF or in PSTAIR immunoprecipitates (Gautier et al., 1988). These results were exciting because the same protein had been identified as a central regulator of the cell cycle and M phase by two independent approaches, genetics in yeast and biochemistry in *Xenopus*. It also indicated the mitotic role of $p34^{cdc2}$ was as a subunit of the MPF complex. In addition, it provided an immediate potential regulatory system for MPF in the light of the extensive network of other genetically identified regulatory elements affecting *cdc2* (Lee & Nurse, 1988), some of which were described earlier.

Properties and Regulation of cdc2 Protein Kinase. Biochemically, *cdc2* protein kinase has several unusual properties. It is only the second serine/threonine protein kinase able to utilize both ATP and GTP, and γ-S-ATP is also a substrate. Although β-glycerophosphate was important for extraction and stabilization of MPF activity, this compound is the most potent inhibitor yet found of the kinase activity (Erikson & Maller, 1989b). The kinase can utilize as divalent cations both Mg^{2+} and Mn^{2+} with optima of 10 and 0.2 mM, respectively. Analysis of MPF has revealed a very narrow substrate specificity, with histone H1 being the best substrate so far identified. α-Casein and phosphatase inhibitor 1 are phosphorylated at less than 5% the rate of H1, and MAP-2 is phosphorylated at 40% the rate of H1. *cdc2* protein kinase is unable to phosphorylate glycogen synthase, unlike most previously characterized serine/threonine protein kinases. Analysis of the sites in histone H1 phosphorylated by *cdc2* kinase has revealed that they are the so-called growth-associated sites involved in chromatin condensation, which become stoichiometrically phosphorylated in mitosis (Allan et al., 1982; Langan, 1982). A growth-associated kinase purified from chromatin fractions of Novikoff heptoma cells on the basis of phosphorylation of these sites has been found to contain $p34^{cdc2}$ complexed with a 62-kDa protein (Langan et al., 1989). Several other investigators have also reported identification of an "M phase specific" histone H1 kinase that contains $p34^{cdc2}$ with one or more additional components (Arion et al., 1988; Meijer et al., 1989; Brizuela et al., 1989). Whether these cytosol-derived kinase complexes exhibit MPF activity is not certain at present, but they have appreciable kinase activity in interphase, as originally reported for chromatin-derived mammalian growth-associated kinase (Langan, 1982), indicating they are not really M phase "specific" but do undergo a severalfold activation in M phase.

Analysis of the phosphorylation sites in H1 histone that are phosphorylated by *cdc2* kinase has revealed a consensus sequence of $(^{K}/_{R})^{S}/_{T}P(X)^{K}/_{R}$, where the elements in parentheses are present in some but not all sites (Langan et al., 1980). Given the rather narrow substrate specificity of *cdc2* kinase, a computer search was carried out of 5251 known sequences, which identified 7% as having at least one consensus site. Many of these proteins turn out to be protooncogenes, including $pp60^{c\text{-}src}$, c-*myc*, c-*myb*, and retinoblastoma-associated protein, and many are also DNA-binding proteins (Table I). Some of these putative substrates are more likely to be involved in DNA synthesis and thus the G_1/S function of $p34^{cdc2}$. The likelihood that MPF carries out phosphorylation at these sites has been shown directly in the case of $pp60^{c\text{-}src}$. In vitro, both immunoprecipitated and baculovirus-produced $pp60^{c\text{-}src}$ are phosphorylated by *cdc2* kinase at the consensus sites (Shenoy et al., 1989; Morgan et al., 1989). These are the same sites specifically phosphorylate in mitosis in fibroblasts, when $pp60^{c\text{-}src}$ kinase activity is 4–7-fold activated and the protein undergoes a pronounced shift in electrophoretic mobility (Chackalaparampil & Shalloway, 1988). At present, however, it has not been shown that phosphorylation by *cdc2* kinase is sufficient to activate $pp60^{c\text{-}src}$ in vitro. The phosphorylation of sites in other substrates not containing this consensus is likely, inasmuch as RNA polymerase II has been reported to be phosphorylated by a homologue of $p34^{cdc2}$ at sites remote from any basic residue, although the motif of proline C-terminal to serine or threonine is conserved (Cisek & Gordon, 1989). Clearly the identification of additional substrates, particularly protooncogenes, merits urgent attention. These results are exciting because they provide a direct link between cell cycle control and cell growth control by protooncogenes. One could speculate that the changes in cell/cell interactions and cytoskeletal structure in mitosis may be mediated by protooncogenes activated by MPF, inasmuch as several structural features of normal mitotic cells resemble those seen in transformed cells (Cooper, 1989; Warren & Nelson, 1987). These studies also raise the question of when in the cell cycle oncogenes exert their regulatory roles. It has been assumed such roles are most evident in the G_0/G_1 transition, but examination of mitotic roles now seems warranted.

Table I: Subset of Proteins with *cdc2* Kinase Consensus Sites

$pp60^{src}$	RB-associated protein	*c-*myc*
D-*src28*	polyoma mT	*c-*myb*
E1b	*Hep B DNA polym[a]	*polyoma lgT
E2A	*HIV polym	*SV40 lgT
mos	*HTLV-II trans-activating protein	*JC lgT

[a] An asterisk indicates a DNA-binding protein.

As mentioned earlier, when MPF is injected into oocytes, a large burst in protein phosphorylation occurs, encompassing a 3–5-fold increase in the total amount of protein phosphate (Maller et al., 1977). This pleiotropic increase is likely to be reflective of the metabolic and structural reprogramming of the cellular biochemistry for mitosis. At present only three substrates have been identified as undergoing phosphorylation—lamins, nucleoplasmin, and ribosomal protein S6 (Miake-Lye & Kirschner, 1985; Neilsen et al., 1982; Cotten et al., 1986). In the case of S6, it has been shown that, upon MPF injection, S6KII is activated by phosphorylation on serine and threonine residues to the same extent as seen with progesterone (Erikson & Maller, 1989a). This activation may represent part of the mechanism by which MPF increases protein synthesis 2-fold (Wasserman et al., 1982). However, S6KII is also able to phosphorylate lamins at mitotic site(s), suggesting S6KII activation could be important for disassembly of the nuclear envelope (Erikson & Maller, 1988; Ward & Kirschner, 1989). Extended analysis shows that S6KII activity against both S6 and lamins oscillates in the cell cycle with kinetics similar to those of MPF. Whether the pathway for S6KII activation is via the MPF-dependent phosphorylation and activation of $pp60^{c\text{-}src}$ remains to be determined. However,

in vitro MPF is unable to phosphorylate either MAP kinase or S6KII, indicating that there are additional intermediates between MPF and MAP kinase in the pathway.

Identification of p45 as a B-Type Cyclin. An important question concerns the nature of the other subunit of purified MPF, p45. A leading candidate was one of the cyclins. Cyclins were first identified in sea urchin eggs as proteins that accumulated continuously during interphase but were quantitatively degraded at the metaphase → anaphase transition during mitosis (Evans et al., 1983; Standart et al., 1987; Murray & Kirschner, 1989). Sequence analysis of cyclins from several species has shown they fall into two classes, A and B, on the basis of sequence similarity. Direct evidence that cyclins played a role in mitotic regulation came from the finding that synthetic mRNA for cyclins from various species could induce maturation when injected into *Xenopus* oocytes (Swenson et al., 1986; Pines & Hunt, 1987). Suggestive evidence for interaction of cyclins with *cdc2* protein came from the findings that small amounts of cyclin could be found in $p34^{cdc2}$ immunoprecipitates and that cyclin antibodies coprecipitated a histone H1 kinase activity (Draetta et al., 1989). In general, cyclins from different species are divergent in sequence except for a prototypic "cyclin box" motif found in the middle third of many cyclin molecules. Hunt and coworkers used oligonucleotide probes against this region to isolate *Xenopus* cDNAs for two B-type cyclins (Minshull et al., 1989) termed B1 and B2. Ablation of these mRNAs in *Xenopus* egg cell-free extracts caused arrest of the cell cycle. Western blotting and immunoprecipitation experiments with antibodies to cyclin B2 showed that it corresponded to p45 in purified MPF, and cyclin B1 was also present in similar amounts (Gautier et al., 1990). A B-type cyclin was also found to be the second subunit of MPF purified from starfish oocytes (Labbe et al., 1989b; Meijer et al., 1989). Both cyclins B1 and B2 could be phosphorylated in vitro by purified MPF, and cell cycle analysis showed the kinase activity against cyclin oscillated with kinetics identical with those of $p34^{cdc2}$. These results are exciting because they complete the molecular identification of MPF and provide an immediate control mechanism to regulate MPF activity via degradation of the cyclin component at the metaphase → anaphase transition. As mentioned earlier, in oocytes injected with catalytic amounts of MPF, a several hundred fold activation of MPF occurs. This result implies that cyclin B2 should be already present in resting G_2 phase oocytes, and Western blotting experiments show that cyclin B2 is indeed present in resting oocytes (Gautier et al., in preparation). A similar situation in meiosis I has also been described in surf clam eggs (Swenson et al., 1986). Although amounts of *cdc2* protein do not change during the cell cycle in various cell types, it thus appears meiosis I is unusual among cell cycle phases in containing a stored reservoir of cyclin as well. This result raises the question of why protein synthesis is needed for meiosis I in *Xenopus*, since both subunits of MPF are present and already associated as "pre-MPF" in resting oocytes (Gautier et al., in preparation). Recent evidence indicates the synthesis of the protooncogene c-*mos* increases severalfold early after mitogenic stimulation (Sagata et al., 1988, 1989; Freeman et al., 1989). Antisense oligonucleotides directed against c-*mos* block activation of MPF and S6 kinase (Barrett et al., 1990), and injection of synthetic c-*mos* mRNA induces maturation in the absence of mitogenic stimuli (Sagata et al., 1989). These results suggest the hypothesis that c-*mos* is involved in the activation of pre-MPF in the oocyte.

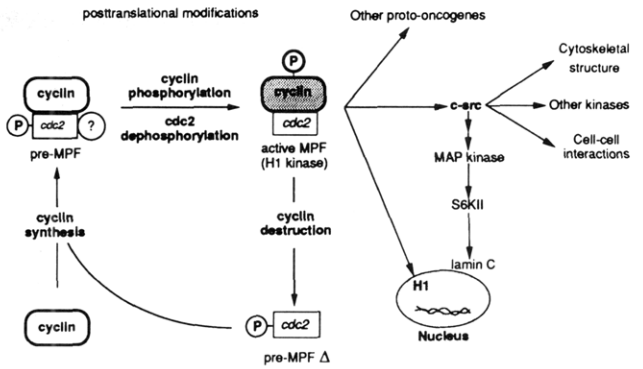

FIGURE 4: Working model of cell cycle control by MPF. The model shows that MPF activity is regulated by synthesis and degradation of the cyclin subunit and phosphorylation/dephosphorylation of the $p34^{cdc2}$ subunit. Other possible regulatory features are phosphorylation of cyclin and association with other components, but this is not yet clear. The mechanism of action of MPF to cause nuclear events can be accounted for in part by direct phosphorylation and activation of other kinases such as $pp60^{c\text{-}src}$. Since S6KII is regulated indirectly by $pp60^{v\text{-}src}$ in transformed cells, it is possible but not yet proven that the MPF-dependent but indirect activation of S6KII is via phosphorylation and activation of the c-*src* protooncogene.

Regulation of $p34^{cdc2}$ Kinase Activity. It seems clear that the cyclin subunit of MPF is regulated by its synthesis and degradation. It is likely also to be regulated by phosphorylation by $p34^{cdc2}$ and other kinases, perhaps including c-*mos*. In the case of $p34^{cdc2}$ kinase, two general mechanisms have been identified for regulating its activity (Figure 4). One is phosphorylation/dephosphorylation, and the other is formation of complexes with other proteins. Draetta and Beach (1988) showed that during G_2 phase in HeLa cells a small fraction of *cdc2* protein (5%) entered into a 170-kDa complex with a 62-kDa protein (most likely a cyclin) and the 13-kDa product (p13) of the *sucI* gene. However, only a small fraction of *sucI* protein in the cell is bound to *cdc2* protein (Draetta & Beach, 1988). Experimentally, the heat-stable *sucI* protein is valuable because when bound to beads it can remove all $p34^{cdc2}$ from extracts and does not appear to markedly inhibit kinase activity of preactivated $p34^{cdc2}$ (Dunphy & Newport, 1989). Thus, although many proteins in addition to $p34^{cdc2}$ are brought down by $p13^{sucI}$ beads (Dunphy et al., 1988), the beads can be used to monitor changes in total *cdc2* kinase activity more conveniently than immunoprecipitation. However, since only a small fraction of $p34^{cdc2}$ is present in various complexes, the *cdc2* protein bound to $p13^{sucI}$ beads may be derived from more than one complex or contain both inactive monomer and active complexed $p34^{cdc2}$, complicating the interpretation of the results. Elution of *cdc2* protein from $p13^{sucI}$ beads requires either SDS or soluble *sucI* protein (Dunphy et al., 1988; Labbe et al., 1989b); elution with the latter protein has been proposed as an aid in the purification of MPF.

Several different complexes of $p34^{cdc2}$ have been identified in cells by either purification or coprecipitation with antibodies or $p13^{sucI}$ beads (Lohka et al., 1988; Gautier et al., 1988; Brizuela et al., 1987; Draetta & Beach, 1988, Wittenberg & Reed, 1988). In most of those cases there is evidence to suggest at least one of the components is cyclin. However, it is not certain if all of these complexes represent MPF activity. At present MPF has been purified from two sources, and in both cases it consists only of a B-type cyclin complexed with $p34^{cdc2}$ with no detectable *sucI* protein (Lohka et al., 1988; Labbe et al., 1989b). This suggests that $p13^{sucI}$-containing complexes identified by immunoprecipitation have another function, although it is possible that *sucI* protein participates in early steps of MPF activation and is then removed.

The other major mechanism regulating *cdc2* protein kinase activity is phosphorylation/dephosphorylation. Draetta and Beach (1988) first reported that human *cdc2* protein immunoprecipitated in a high molecular weight complex was phosphorylated, and they suggested phosphorylation underlay the activation process. However, more careful studies with more highly synchronized cells showed that in fact just prior to entry into M phase mouse *cdc2* protein became *de*phosphorylated (Morla et al., 1989). There is now a very good correlation in several cell systems between phosphorylation and kinase inactivation of *cdc2* protein upon exit from M phase and between dephosphorylation and activation of kinase activity during entry into M phase (Gautier et al., 1989; Dunphy & Newport, 1989; Morla et al., 1989). This biochemical finding is exciting because of the previous genetic work in *S. pombe* showing that the timing of mitosis-specific *cdc2* kinase functions was delayed by the expression of the *wee1*$^+$ gene, which encodes a serine/threonine protein kinase (Russell & Nurse, 1987a). This is another example of the convergence of genetics and biochemistry in the studies of cell cycle regulation.

p34^{cdc2} is only the second example of a serine/threonine protein kinase that is inactivated by phosphorylation, the classic case being the phosphorylation and inactivation of myosin light chain kinase by cAMP-dependent protein kinase (Conti & Adelstein, 1981). Phosphoamino acid analysis of *cdc2* protein from interphase has shown it is phosphorylated on both tyrosine and threonine residues (Morla et al., 1989), and anti-phosphotyrosine antibodies have also been used to monitor changes in phosphotyrosine content (Dunphy & Newport, 1989). This suggests two different signaling systems are likely to be involved in p34^{cdc2} inactivation, one a tyrosine kinase pathway and the other a threonine kinase pathway. However, the significance of the tyrosine phosphorylation of p34^{cdc2} in vertebrate cells is unclear at present. Beach and collaborators (Draetta et al., 1988) originally reported that human *cdc2* protein was a substrate for pp60$^{c\text{-}src}$, a particularly interesting possibility in light of the phosphorylation of *src* protein by *cdc2* kinase (Shenoy et al., 1989; Morgan et al., 1989). However, the study by Draetta et al. (1988) used inactive denatured *cdc2* protein, and native p34^{cdc2} does not appear to be a substrate for pp60$^{c\text{-}src}$ (Shenoy et al., 1989; Morgan et al., 1989), although after denaturation it does become a substrate (J. L. Maller, unpublished). Inactivation by phosphorylation is likely to be a conserved feature since p34^{cdc2} is also tyrosyl- and threonyl-phosphorylated in *S. pombe* (Gould & Nurse, 1989), where tyrosyl-phosphorylated proteins have been difficult to find. Dunphy and Newport (1989) showed that the *sucI* protein blocked the tyrosyl dephosphorylation of pre-MPF from oocytes and prevented activation of its kinase activity and ability to cause nuclear breakdown. Once p34^{cdc2} was activated, however, *sucI* protein bound equally well to p34^{cdc2} but had no effect on kinase activity, a property that makes p13^{sucI} beads a particularly valuable tool for monitoring changes in p34^{cdc2} kinase activity during the cell cycle. Whether the *sucI* protein also prevents threonine dephosphorylation was not investigated, since all analysis was by anti-phosphotyrosine antibody blotting. However, it seems likely that *sucI* protein also blocks threonine removal, since tyrosine dephosphorylation of mouse p34^{cdc2} does not cause activation (Morla et al., 1989) and both phosphorylated residues are adjacent. In spite of the very strong correlation between phosphorylation and inactivation of *cdc2* kinase, isolation of the kinases and phosphatases that act on p34^{cdc2} is required to establish that phosphorylation/dephosphorylation is causally responsible for changes in enzyme activity. Identification of the tyrosine kinase involved may be most efficient in yeast, due to the great scarcity of phosphotyrosyl proteins and presumably kinases in this cell and to the power of genetic analysis.

Figure 4 presents a pictorial view of the current level of understanding of MPF regulation and action. This picture is likely to be outdated rapidly because of the remarkable rate of progress recently in characterizing MPF. The model shows a cycle in which MPF activity is regulated by the synthesis and degradation of the cyclin subunit as well as phosphorylation of cyclin by *cdc2* kinase and possibly other kinases. The model also suggests that the association of p34^{cdc2} with cyclin, and possibly other (?) proteins, is a regulated event, although no evidence is currently available on this point. p34^{cdc2} is depicted as undergoing regulation by phosphorylation/dephosphorylation on both threonine and tyrosine residues. Targets of active MPF are shown as histone H1, at sites believed to be crucial for chromosome condensation, and pp60$^{c\text{-}src}$, which may be involved in the pleiotropic change in the cytoskeleton and cell/cell interactions that occur in mitosis. The bulk of the MPF-induced burst of phosphorylation occurs on proteins that are not direct substrates for MPF, such as ribosomal protein S6 and lamins. The figure suggests S6KII is a downstream kinase activated by MPF for both S6 and lamin phosphorylation, possibly via activation of pp60$^{c\text{-}src}$, MAP kinase kinase, and MAP kinase, although this has not yet been established. Presumably the early increase in S6KII activity occurring immediately after inactivation of the insulin receptor kinase does not involve MPF and represents only those elements of the signaling pathway distal to tyrosine kinase activation.

In summary, the *Xenopus* oocyte and egg system has been instrumental in defining steps in tyrosine kinase signaling pathways and in developing a cell-free system able to carry out cell cycle traverse in vitro. Use of this system has led to molecular elucidation of MPF and identified links between cell cycle control and cell growth control by protooncogenes. It is likely that further insights into the biochemistry of cell proliferation will come from analysis of oocyte maturation and of MPF in the cell-free system.

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Accelerated Publications

α- and β-Forms of the 65-kDa Subunit of Protein Phosphatase 2A Have a Similar 39 Amino Acid Repeating Structure[†,‡]

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ABSTRACT: Protein phosphatase 2A (polycation-stimulated protein phosphatase L) was purified from porcine kidney and skeletal muscle. The 36-kDa catalytic and the 65-kDa putative regulatory (hereafter termed PR65) subunits of protein phosphatase 2A2 were separated by reverse-phase HPLC. Partial amino acid sequence data (300 residues) was obtained for PR65. Molecular cloning showed that two distinct mRNAs (termed α and β) encoded the PR65 subunit. The cDNA encoding the α-isotype spanned 2.2 kilobases (kb) and contained an open reading frame of 1767 bases predicting a protein of 65 kDa, which was in good agreement with the size of the purified protein. The cDNAs encoding the β-isotype contained an open reading frame of size similar to that of α-form but lacked an initiator ATG. Northern analysis, using RNA isolated from several human cell lines, indicated that the α-isotype was encoded by a mRNA of 2.4 kb that was much more abundant than the β mRNA of 4.0 kb. Comparison of the predicted amino acid sequences of the two isotypes revealed 87% identity. The deduced protein sequences of the α- and β-isotypes were found to be made up of 15 imperfect repeating units consisting of 39 amino acids. This repeating structure was conserved between species.

Protein phosphorylation plays a central role in regulating a diverse number of cellular processes. For phosphorylation of specific target proteins to function as an effective and dynamic control mechanism, it requires the coordinated action of both protein kinases and phosphatases. The role of hormones, growth factors, andd neurotransmitters in regulating protein kinase activity has been extensively investigated [reviewed by Edelman et al. (1987) and Yardin and Ullrich (1988)]. However, the influence of different signal transduction pathways on the regulation of protein phosphatases is less well established.

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