

## Activation of p34<sup>cdc2</sup> Kinase by Cyclin A

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**Abstract.** Functional clam cyclin A and B proteins have been produced using a baculovirus expression system. Both cyclin A and B can induce meiosis I and meiosis II in *Xenopus* in the absence of protein synthesis. Half-maximal induction occurs at 50 nM for cyclin A and 250 nM for cyclin B. Addition of 25 nM cyclin A to activated *Xenopus* egg extracts arrested in the cell cycle by treatment with RNase or emetine activates *cdc2* kinase to the normal metaphase level and stimulates one oscillatory cell cycle. High levels of cyclin A cause marked hyperactivation of *cdc2* kinase

and a stable arrest at the metaphase point in the cell cycle. Kinetic studies demonstrate the concentration of cyclin A added does not affect the 10 min lag period required for kinase activation or the timing of maximal activity, but does control the rate of deactivation of *cdc2* kinase during exit from mitosis. In addition, exogenous clam cyclin A inhibits the degradation of both A- and B-type endogenous *Xenopus* cyclins. These results define a system for investigating the biochemistry and regulation of *cdc2* kinase activation by cyclin A.

RECENTLY, there has been significant progress in understanding control of the cell cycle restriction point governing transit from G<sub>2</sub> phase into M phase (Pines and Hunter, 1990, for review). Genetically, studies in the fission yeast *Schizosaccharomyces pombe* demonstrated that the *cdc2*<sup>+</sup> gene, which encodes a 34-kD serine/threonine protein kinase, was a major locus of control over the G<sub>2</sub> → M transition (Nurse and Bisset, 1981; Simanis and Nurse, 1986; Nurse et al., 1976). The human homologue of *cdc2*<sup>+</sup> was cloned by complementation in *S. pombe*, providing evidence of functional conservation of cell cycle control elements over a billion years of evolution (Lee and Nurse, 1987). Biochemically, the product of the *cdc2*<sup>+</sup> gene, designated p34<sup>cdc2</sup>, was found to be the protein kinase subunit of purified maturation-promoting factor (MPF)<sup>1</sup> (Gautier et al., 1988; Dunphy et al., 1988). MPF had been identified many years ago as an activity able to cause entry into mitosis or meiosis in vivo or in vitro in a variety of systems, suggesting it was a fundamental regulatory element (Masui and Markert, 1971; Kishimoto et al., 1982). As purified, MPF contains p34<sup>cdc2</sup> complexed with a B-type cyclin (Gautier et al., 1990; Labbé et al., 1989). Cyclins were first identified in fertilized marine invertebrate eggs as proteins that accumulated during interphase but were then quantitatively degraded near the metaphase/anaphase transition (Evans et al., 1983; Standard et al., 1987). Subsequent studies have shown that cyclins fall into two classes, designated A and B, that can be distin-

guished by sequence similarity as well as by their kinetics of degradation in the cell cycle (Westendorf et al., 1989; Minshull et al., 1989, 1990). B-type cyclins are related in sequence to the *cdcl3*<sup>+</sup> gene in *S. pombe*, whose product is known to form a complex with p34<sup>cdc2</sup> and to be degraded at metaphase (Booher and Beach, 1988; Solomon et al., 1988; Goebel and Byers, 1988; Hagan et al., 1988).

Many aspects of cell cycle control appear to be explained by changes in the abundance or activity of the cyclin component, which in turn is thought to affect the protein kinase activity of p34<sup>cdc2</sup> by mechanisms that include stimulation of the phosphorylation and dephosphorylation of the kinase itself on both tyrosine and threonine residues (Solomon et al., 1991). Interest in the action of cyclin came originally from the finding that injection of synthetic mRNA for clam cyclin A or B into *Xenopus* oocytes stimulated the G<sub>2</sub> → M transition (Swenson et al., 1986; Westendorf et al., 1989). Because of the regulated nature of cyclin abundance in fertilized marine eggs, it seemed likely from these results that the synthesis of cyclin represented the well-documented protein synthesis requirement for meiosis I and II in *Xenopus* (Wasserman and Masui, 1975; Gerhart et al., 1984). However, subsequent studies by Sagata, Vande Woude, and co-workers and others have demonstrated that increased synthesis of the *mos*<sup>sc</sup> protooncogene, which encodes a serine/threonine protein kinase, is both necessary and sufficient for meiotic cell cycles in *Xenopus* (Sagata et al., 1988, 1989a; Freeman et al., 1989; Barrett et al., 1990). Consistent with a requirement for the synthesis of *mos*<sup>sc</sup> during the meiotic cycles of oocyte maturation and the amplification of MPF activity

1. **Abbreviations used in this paper:** CSF, cytotostatic factor; MPF, M-phase promoting factor; PMSG, pregnant mare's serum gonadotropin.

in cycloheximide-treated oocytes (Wasserman and Masui, 1975), recent work demonstrates that cyclin B<sub>2</sub> preexists in resting G<sub>2</sub> phase oocytes and is already partially phosphorylated and associated with p34<sup>cdc2</sup> in an inactive complex called pre-MPF (Gautier and Maller, 1991). In extracts of activated eggs, B-type cyclins, and *mos*<sup>src</sup> kinase are thought to be degraded at exit from M phase (Murray and Kirschner, 1989; Watanabe et al., 1989), and activation of p34<sup>cdc2</sup> kinase in the first mitotic cell cycle is dependent on the synthesis of cyclin B (Minshull et al., 1989; Murray and Kirschner, 1989). Expression in extracts of nondegradable forms of cyclin B leads to a stable arrest in metaphase, and a high level of H1 protein kinase activity of p34<sup>cdc2</sup> (Murray et al., 1989).

In nearly all this previous work, the focus has been on cyclin B rather than cyclin A, and functional cyclin B has been produced by translation of synthetic mRNA in cells or in extracts. A number of important questions about the role of cyclin A merit urgent attention. These questions include whether cyclin A mRNA or protein can drive mitotic cell cycles in the absence of cyclin B or other new proteins. Is the mechanism whereby cyclin A activates p34<sup>cdc2</sup> kinase the same as cyclin B? We believe these questions are difficult to answer when expressing cyclin by translation of synthetic mRNA because the concentration of cyclin is continuously changing throughout the experiment. To begin to address these questions biochemically, we have used insect cells (Sf9) infected with recombinant baculovirus encoding clam cyclin A and B genes to produce functional cyclin proteins, permitting us to carry out experiments with defined concentrations of cyclin without the need for protein synthesis. In this paper we have compared these cyclins for their ability to stimulate meiotic cell cycles and probed the mechanism of activation of p34<sup>cdc2</sup> kinase by cyclin A in mitotic cycles.

## Materials and Methods

### Preparation of Murray and Cytostatic Factor (CSF) Extracts

Female *Xenopus laevis* (Xenopus 1, Ann Arbor, MI) were primed with 50 and 25 U of PMSG (pregnant mare's serum gonadotropin) 5 and 3 d, respectively, before the experiment. The frogs were then injected with 100 U HCG (human chorionic gonadotropin) 14–16 h before the experiment. The eggs were laid in 1× Maller Modified Ringer's (MMR) (110 mM NaCl, 1.8 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 5 mM Na Hepes, pH 7.8). Oscillating extracts were prepared as described by Murray and Kirschner (1989) with several modifications. Instead of electrical activation, the eggs were activated by a 60–80-s treatment in 0.2× MMR with the calcium ionophore A23187 at 5 μg/ml of eggs, followed by at least ten washes with 0.2× MMR. At the times indicated in the figures, aliquots of the oscillating extract were taken and diluted 20 times with distilled H<sub>2</sub>O to arrest cycling, immediately frozen in dry ice, and later assayed for histone H1 kinase activity. In some experiments aliquots of extracts were visualized by fluorescence microscopy to ascertain nuclear morphology, as described previously (Lohka and Maller, 1985). To inhibit oscillation of the cell cycle, extracts were treated at 10°C for 20 min with 0.3 μg/ml DNase-free ribonuclease (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Placental ribonuclease inhibitor, RNasin (Promega Biotec, Madison, WI) was then added to a final concentration of 1,500 U/ml, and the extracts were further incubated for 15 min at 10°C. Alternatively emetine was added to 100 μM to inhibit protein synthesis. Extracts were incubated at 23°C during oscillations. Assays at 0 time represent samples measured just before incubation at 23°C. CSF-arrested extracts were prepared according to the protocol for oscillating extracts with the following modifications. Before being crushed, the eggs were washed twice with extract buffer (10 mM K-Hepes, pH 7.7, 100 mM KCl, 0.1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 50 mM Sucrose) containing 5 mM EGTA and a further 1 mM MgCl<sub>2</sub>. The eggs were not activated before being

crushed and thus the extracts remained arrested in M phase until treatment with 0.2 mM CaCl<sub>2</sub> released them into interphase (Lohka and Maller, 1985).

### Histone H1 Kinase Activity

Histone H1 kinase assays were carried out as previously described (Gautier et al., 1989). The samples were analyzed by SDS-PAGE (Laemmli, 1970), excision of histone H1 band, and determination of incorporated radioactivity by Cherenkov counting. The conditions under which these assays were carried out have been previously shown to be specific for p34<sup>cdc2</sup> kinase as judged by analysis of sites phosphorylated in histone H1 as well as by the lack of effect of known inhibitors of other H1 kinases (Langan et al., 1989). In addition, oscillatory behavior reported here has also been observed with kinase activity adsorbed to p13<sup>SUC1</sup> Sepharose beads, a reagent with high affinity for p34<sup>cdc2</sup>.

### Isolation of *Xenopus cdc2*/Cyclin Complexes on p13<sup>SUC1</sup>

p13<sup>SUC1</sup> was purified from an overproducing *Escherichia coli* BL21 (DE3) strain after a procedure modified from Brizuela et al. (1987). The p13<sup>SUC1</sup> protein was coupled to activated CH-Sepharose CL-4B beads (Pharmacia Fine Chemicals) at 4°C for 1 h at pH 6.5, with a final concentration of *SUC1* on the bead of 5 mg/ml of resin. To adsorb the *cdc2*/cyclin complexes from the *Xenopus* extracts, 5-μl samples were diluted with 150 μl of buffer containing 20 mM Hepes, pH 7.5, 15 mM MgCl<sub>2</sub>, 20 mM EGTA, 15 mM DTT, 80 mM βGP, 0.5 mM PMSF, 3 μg/ml leupeptin, 50 mM NaF, 1 mM sodium vanadate, 0.2 mM ammonium molybdate, 30 mM *p*-nitrophenol phosphate, and precleared with 50 μl of 50% Sepharose beads. After centrifugation at 16,000 g for 5 min, the supernatant was transferred to fresh tubes and mixed with 25 μl of a 50% p13-Sepharose suspension for 1 h. The isolated complexes were prepared for the H1 kinase assay by washing the beads with a buffer containing 20 mM Tris pH 7.4, 5 mM EDTA, 100 mM NaCl, 1% TX-100, twice with the same buffer containing 1 M NaCl, and finally twice with the H1 kinase buffer. The <sup>35</sup>S-labeled complexes were washed three times with RIPA buffer (20 mM Tris pH 7.4, 5 mM EDTA, 100 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40), twice with RIPA containing 1 M NaCl, and twice more with RIPA buffer. The p13<sup>SUC1</sup> precipitates were analyzed by electrophoresis on 10% Anderson gels (Anderson et al., 1973) and fluorography.

### Cell Lines and Generation of Recombinant Baculovirus

*Spodoptera frugiperda* (Sf9) cells were obtained from the American Type Tissue Culture Collection (Rockville, MD). Sf9 cells were cultured in Grace's Antheraea medium supplemented with 10% FBS, 3.3 g/liter of yeastolate, 3.3 g/liter of lactalbumin hydrolysate, and 50 μg/ml gentamicin at 27°C. All procedures relating to viral propagation, isolation, and plaqueing were performed as suggested by Summers and Smith (1987). pCD102 containing the gene encoding clam cyclin B (Westendorf et al., 1989) was linearized with HindIII and a BglII linker was inserted. The resulting plasmid was then linearized with XbaI and a BglII linker was inserted to generate pCD102(BglII). pCD102(BglII) was then digested with BglII and the 1.7-kb insert containing the cyclin B gene was cloned into the BamHI site of pVL941 (Luckow and Summers, 1988) to generate pVL941(B). pAXH(+) containing the gene encoding clam cyclin A (Swenson et al., 1986) was linearized with HindIII and a BamHI linker inserted. The resulting plasmid was digested with BamHI and the 1.5-kb fragment containing the cyclin A gene was cloned into the BamHI site of pVL941 to generate pVL941(A).

Sf9 cells were transfected with a mixture of 1 μg of purified wild-type viral DNA and 2 μg of either pVL941(A) or pVL941(B). 5 d after transfection, medium supernatants containing both wild-type and recombinant viruses were collected and plaqued onto a monolayer of Sf9 cells. Recombinant viruses were identified by their occlusion negative phenotype and were isolated by plaque purification as described previously (Piwnicka-Worms et al., 1990). Recombinant proteins were detected by immunoblot analysis (Fig. 1).

To generate active cyclin lysates, baculovirus-infected cells were sonicated five times (5-s bursts) using a Kontes microultrasonic cell disrupter (Vineland, NJ) in a buffer containing 20 mM Tris-HCl (pH 7.5) 50 mM NaCl, and 1 mM DTT. In some experiments extracts were clarified by centrifugation at 15,000 g for 10 min. In dose-response experiments the baculovirus-produced cyclin was diluted with the same buffer and added to a final volume of 50 μl of the oscillating extract. Control additions used ly-

sates of Sf9 cells with wild-type baculovirus. Similar results were obtained in at least three independent experiments with multiple preparations of cyclins. Cyclin activity was stable to freeze-thawing, and experiments comparing potencies of cyclins A and B in oocytes utilized aliquots of the same lysates. Quantitation of cyclin A and B content in Sf9 cell lysates was assessed by densitometric scanning of Coomassie blue-stained gels containing either cyclin lysates at various dilutions or known amounts of glutamate dehydrogenase ( $M_r = 48$  kD). A standard curve of glutamate dehydrogenase densitometric units was then constructed to assess the concentration of cyclin A or B in samples diluted to fit in the linear portion of the standard curve. The stock concentration of cyclin A and B in the lysates used in this paper was  $4 \mu\text{M}$ , constituting  $\sim 10\%$  of the total protein. This is  $\sim 200$  times greater than the effective concentration in extracts, and inasmuch as cyclin was a major Coomassie blue staining band, equivalent to a 2,000-fold purification from eggs, no further purification of cyclin beyond the level achieved by overexpression was carried out. Intracellular concentrations of cyclins after microinjection of 50-nl volumes into oocytes were calculated on the basis of  $0.5 \mu\text{l}$  of cell water per oocyte (Stith and Maller, 1985). In all injection experiments stage VI oocytes from frogs primed 3 d earlier with 35 IU PMSG were manually dissected out of their follicular envelopes on the ovary and cultured in modified medium OR-2 containing 10 mM  $\text{NaHCO}_3$ , pH 7.8.

### Anti-cyclin Antibodies and Immunoblot Analysis

Affinity-purified anti-cyclin A and anti-cyclin B antibodies were produced and characterized as described by Swenson et al. (1986) and Westendorf et al. (1989) respectively. Lysates of insect cells infected with wild-type or cyclin recombinant baculoviruses were dissolved in SDS sample buffer and separated by SDS-PAGE and blotted onto nitrocellulose paper in parallel with samples made from mitotic clam embryos prepared 75 min after fertilization, as described by Swenson et al. (1986). Gel blots were incubated with affinity-purified antibodies, then with an alkaline-phosphatase conjugated secondary antibody (Promega Biotec), and developed according to the manufacturer's instructions.

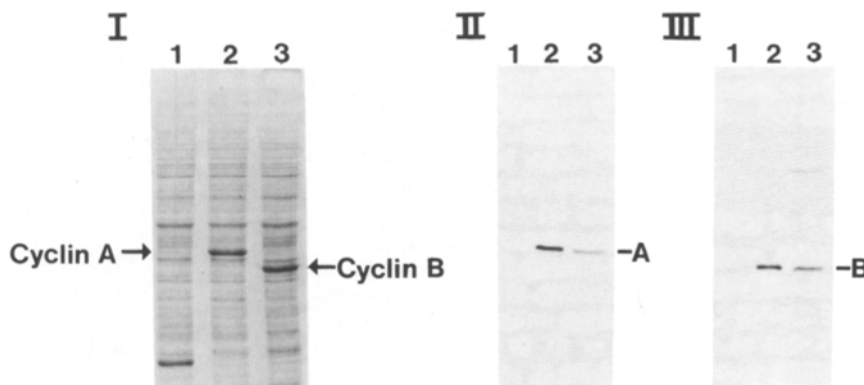
## Results

### Cyclins Stimulate Meiosis I and II in the Absence of Protein Synthesis

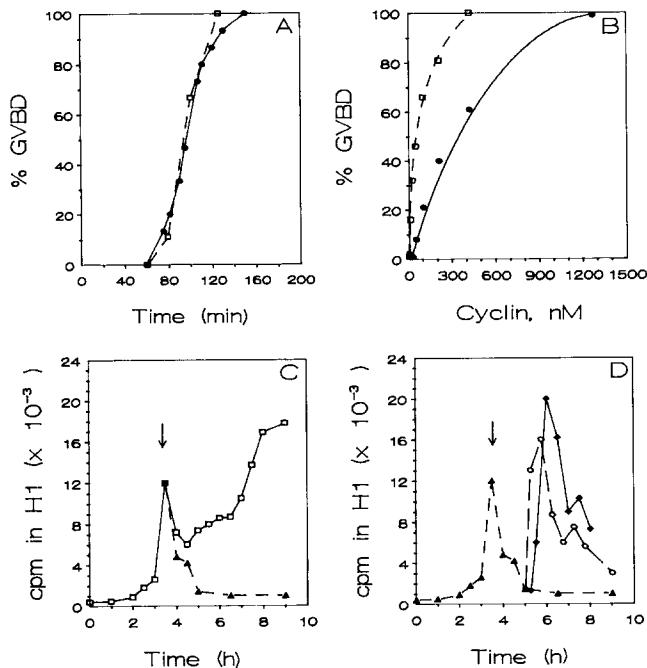
Since all previous reports on the ability of cyclins to stimulate oocyte maturation have used injection of synthetic mRNA for clam cyclin A or B (Swenson et al., 1986; Westendorf et al., 1989), it is unclear whether synthesis of other proteins is required for cyclin-induced oocyte maturation and more specifically, whether the *mos*<sup>sc</sup> kinase is required. To address this question, we overproduced active clam cyclin A and B proteins in Sf9 (*Spodoptera frugiperda*) cells using a

baculovirus expression system. Fig. 1 I shows total lysate proteins prepared from cells infected with wild-type virus or recombinant virus encoding either cyclin A or cyclin B. Immunoblot analysis using anti-cyclin A or B antibodies demonstrated that the prominent overproduced Coomassie blue staining bands were in fact cyclin and that they co-migrated with the cyclin produced in intact clam eggs (Fig. 1, II and III). Analysis of lysates from cells infected with wild-type baculovirus showed neither a prominent Coomassie staining band at the relative molecular mass of cyclin A or B nor any immunoreactive band (Fig. 1). In insect cells, both cyclins associate with coexpressed *cdc2* and activate  $p34^{\text{cdc}2}$  kinase activity to equivalent extents; both complexes are also phosphorylated on tyrosine 15 of *cdc2* kinase when co-expressed with the *wee I*<sup>+</sup> gene (Parker et al., 1991). To determine whether baculovirus-produced cyclins were functionally active in regulating *Xenopus* meiotic cell cycles, we microinjected cyclin A lysates into oocytes in the presence or absence of cycloheximide and monitored germinal vesicle breakdown (GVBD) as a measure of entry into meiosis I. As shown in Fig. 2 A, cyclin A was able to induce GVBD very rapidly (1–2 h), causing 100% GVBD in the presence or absence of cycloheximide. Control injections with lysates of Sf9 cells infected with wild-type virus had no detectable activity. Cycloheximide treatment has previously been shown to reduce *mos*<sup>sc</sup> below levels functional for GVBD (Sagata et al., 1989a). Therefore these results indicate cyclin A-induced maturation meets the criteria for MPF-induced maturation and does not require the *c-mos*<sup>sc</sup> protein kinase or any other newly synthesized protein.

An important question concerns whether cyclins A and B have identical properties in cell cycle control. Therefore, as shown in Fig. 2 B, lysates containing various concentrations of cyclin A and B were microinjected into cycloheximide-treated oocytes, and the frequency of GVBD was assessed 2 h later. Both cyclins were able to induce GVBD, but cyclin B was only about one-fifth as potent as cyclin A. Half-maximal stimulation occurred at 50 nM for cyclin A and 250 nM for cyclin B. Lysates of cells infected with wild-type virus had no effect even when injected at much higher concentrations. Previous work by Gerhart et al. (1984) has shown that protein synthesis is also required for the transition between meiosis I and meiosis II in *Xenopus*. During normal



**Figure 1.** Characterization of clam cyclin A and B expressed in a recombinant baculovirus system. (I) Coomassie blue-stained proteins evident in aliquots of Sf9 cell lysates infected with wild-type virus (lane 1), recombinant cyclin A virus (lane 2), and recombinant cyclin B virus (lane 3). The arrows in lanes 2 and 3 mark the prominent cyclin bands. (II) Samples as in I, lanes 1 and 2, were subjected to Western blotting with anti-cyclin A antibody. Lane 1, wild-type; lane 2, fertilized clam egg lysate; lane 3, lysate from Sf9 cells infected with virus encoding cyclin A. (III) The same experiment as in II was carried out except anti-cyclin B antibody was used and lane 3 contained lysates of Sf9 cells infected with virus encoding cyclin B.

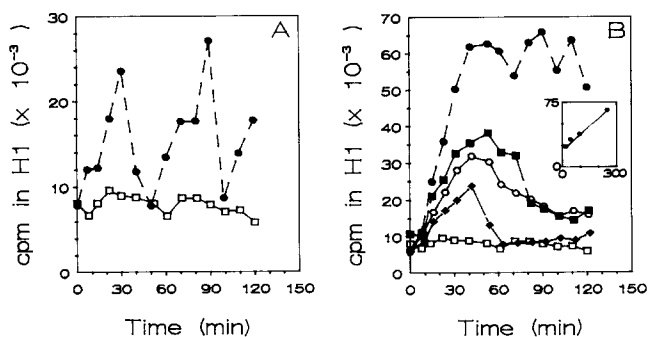


**Figure 2.** Induction of meiosis by cyclins A and B. (A) Clam cyclin A induces GVBD. Oocytes were injected with cyclin A lysates and the rate of germinal vesicle breakdown (GVBD) was assessed. When present, cycloheximide was used at 10  $\mu\text{g}/\text{ml}$  for 30 min before injection as well as during and after microinjection. The final intracellular concentration of cyclin A was 250 nM. Control injections of lysates from cells infected with wild-type virus had no effect.  $\square$ — $\square$ , with cycloheximide;  $\bullet$ — $\bullet$ , without cycloheximide. (B) Quantitative comparison of cyclin A and B for GVBD. Oocytes were incubated in medium OR-2 containing 10  $\mu\text{g}/\text{ml}$  cycloheximide for 30 min before injection as well as during and after injection. Oocytes were injected to the indicated final intracellular concentration of either cyclin A or B in Sf9 cell lysates and the frequency of GVBD assessed after 2 h. Half-maximal induction occurred at 50 nM for cyclin A ( $\square$ — $\square$ ) and 250 nM for cyclin B ( $\bullet$ — $\bullet$ ). No GVBD occurred with lysates of wild-type infected cells at concentrations corresponding to extracts containing 1,200 nM cyclin. (C) Effect of cycloheximide on p34<sup>cdc2</sup> kinase in meiosis II. Oocytes were treated with progesterone (10  $\mu\text{g}/\text{ml}$ ) at zero time and the time course of activation of the H1 histone kinase activity of p34<sup>cdc2</sup> assessed ( $\square$ — $\square$ ). GVBD<sub>50</sub> occurred at 3.5 h. In some cases, oocytes which had undergone GVBD within the previous 10 min (denoted by the arrow) were injected with cycloheximide in OR-2 to give an intracellular concentration of 5  $\mu\text{g}/\text{ml}$  and the time course of p34<sup>cdc2</sup> kinase activity assessed ( $\blacktriangle$ — $\blacktriangle$ ). Control injections of OR-2 had no effect (not shown). (D) Reactivation of meiosis II by clam cyclins A or B. The same experiment as in C is shown for oocytes injected with cycloheximide just after GVBD ( $\blacktriangle$ — $\blacktriangle$ ). At 5 h (cyclin A) or 5.25 h (cyclin B) lysates containing cyclin A ( $\circ$ — $\circ$ ) or cyclin B ( $\blacklozenge$ — $\blacklozenge$ ) were microinjected to give intracellular concentrations of 66 and 200 nM, respectively and the activity of p34<sup>cdc2</sup> kinase assessed for a subsequent 3–4 h period. Control injections of lysates from wild-type baculovirus-infected Sf9 cells had no effect.

progesterone-induced oocyte maturation, the level of MPF activity as measured by the oocyte microinjection assay declines by  $\sim 50\%$  1–2 h after GVBD in conjunction with the metaphase/anaphase transition of meiosis I. This decline in MPF is transient and levels soon return to that seen in a metaphase II-arrested egg; however, if cycloheximide is in-

jected just after GVBD, MPF declines abruptly to undetectable levels and remains low (Gerhart et al., 1984). Fig. 2 C shows that the same effect of cycloheximide can also be measured by assessing the H1 kinase activity of p34<sup>cdc2</sup> rather than MPF activity for GVBD in the microinjection assay.

Studies by Sagata and Vande Woude have shown that the metaphase II arrest of the egg is dependent on expression of *mos<sup>sc</sup>* which appears to have a very short half-life (Sagata et al., 1989b). Therefore, it seems likely the protein synthesis requirement for Meiosis II, like Meiosis I, is also accounted for by synthesis of the *mos<sup>sc</sup>* kinase. Since a possible target for phosphorylation by *mos<sup>sc</sup>* is cyclin B2 in pre-MPF (Roy et al., 1990), it seemed plausible that functional cyclin could rescue a meiosis II cell cycle made defective by the absence of *c-mos<sup>sc</sup>*. To assess this possibility, either cyclin A or B lysates were microinjected into oocytes that had been previously injected with cycloheximide immediately after undergoing progesterone-induced GVBD. As shown in Fig. 2 D, both cyclins caused an immediate increase in the H1 kinase activity of p34<sup>cdc2</sup> in the presence of cycloheximide. At the concentration of cyclin used, this increase reached the same level as in metaphase II-arrested oocytes, but unlike controls, which exhibit a stable metaphase II H1 kinase activity (cf. Fig. 2 A), cyclin-injected oocytes underwent a rapid decline in H1 kinase activity after reaching metaphase II levels, presumably reflecting exit from second meiotic metaphase into interphase. This might be a consequence of the absence of *c-mos<sup>sc</sup>*, which is necessary for metaphase II arrest (Sagata et al., 1989b). This rescue of a cycloheximide-inhibited cell cycle by cyclin occurred at concentrations of cyclin similar to those that caused GVBD and meiosis I. However, at higher concentrations of cyclin A, H1 kinase activity was elevated to levels higher than progesterone controls at GVBD, and H1 kinase activity remained high for a prolonged period (data not shown).



**Figure 3.** Cyclin A drives mitotic cell cycles. (A) Effects of RNase on oscillation of p34<sup>cdc2</sup> kinase. Oscillating extracts were prepared as described in Materials and Methods with ( $\square$ — $\square$ ) or without ( $\bullet$ — $\bullet$ ) treatment with RNase. The RNased extract was supplemented with a wild-type Sf9 cell lysate. (B) Effect of cyclin A on p34<sup>cdc2</sup> kinase. To the same RNased extracts depicted in A, lysates containing various concentrations of cyclin A were added at zero time and the activity of p34<sup>cdc2</sup> kinase assessed over a 2-h period encompassing two oscillations in controls (cf. A). Inset: The maximal amplitude of H1 kinase activity at 45 min is plotted (ordinate) as a function of the final concentration of cyclin A in the extract (abscissa). Units on the axes in the inset are the same.  $\square$ — $\square$ , wild-type;  $\blacklozenge$ — $\blacklozenge$ , 25 nM cyclin A;  $\circ$ — $\circ$ , 50 nM cyclin A;  $\blacksquare$ — $\blacksquare$ , 100 nM cyclin A; ( $\bullet$ — $\bullet$ ), 250 nM cyclin A.

**Table 1. Effect of Cyclin A on Nuclear Morphology**

Minutes	Control	RNase	Cyclin A-treated
0	+	+	+
10	+	+	+
20	+	?	+
30	+	+	+
40	+	+	+
50	+	+	+
60	-	-	+
70	-	-	+
80	+	-	+
90	+	-	+
100	+	-	+
110	-	-	+
120	-	-	+
130	+	-	+

The effect of oscillating extracts on nuclear morphology was determined by adding sperm pronuclei (100,000/ml) to the different extracts at time zero. Samples were taken at different times, stained with DAPI, and examined by phase and fluorescence microscopy to identify the phases of the nuclei. Cyclin A-treated extracts contained 250 nM cyclin A. +, mitotic chromosomes; -, interphase nuclei.

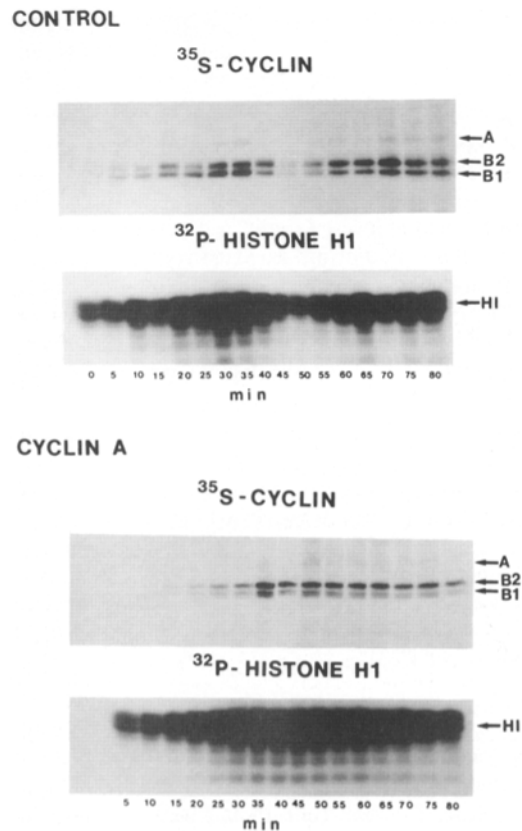
### Cyclin A Activation of p34<sup>cdc2</sup> for Mitotic Cell Cycles

Murray and Kirschner (1989) modified the original *Xenopus* egg extraction protocol of Lohka to produce mitotic extracts that would oscillate spontaneously between DNA replication and mitosis. These oscillatory egg extracts have been called by many investigators Murray extracts to distinguish them from Lohka extracts, which require addition of exogenous components to transit cell cycle restriction points. They demonstrated that in extracts made shortly after egg activation sea urchin cyclin B mRNA alone could drive a *Xenopus* cell cycle from interphase into M-phase, and cyclin B degradation was required to exit M phase (Murray and Kirschner, 1989; Murray et al., 1989). Within a certain range, the length of the cell cycle before the activation of MPF appeared to be dependent on the concentration of newly translated cyclin B added in a reticulocyte lysate, suggesting the amount of cyclin B present controlled the rate of p34<sup>cdc2</sup> kinase activation and cell cycle progression (Murray et al., 1989). However, only a twofold range of active cyclin mRNA was tested for effectiveness in driving the cycle (Murray and Kirschner, 1989).

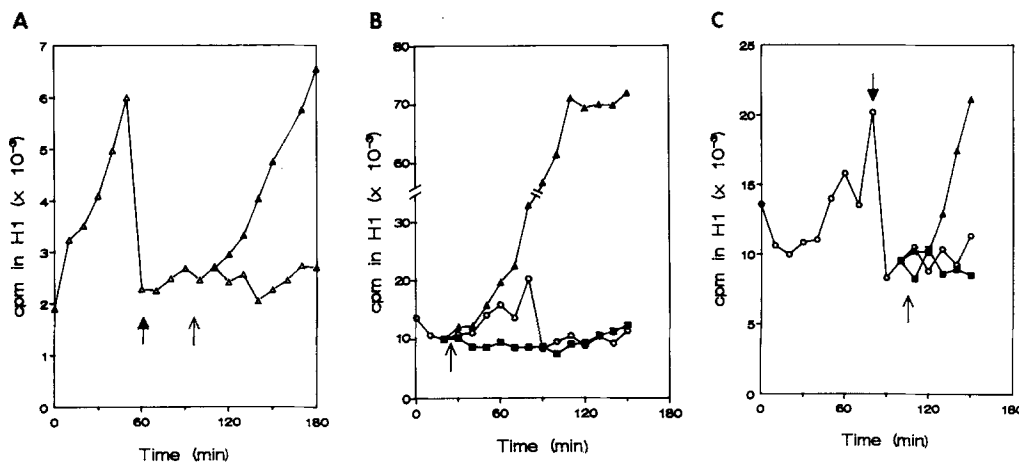
None of the previous work in mitotic extracts has addressed the function of cyclin A. To address whether cyclin A translation was sufficient for entry into mitotic cell cycles, we developed a sensitive and quantitative assay for cyclin A activation of *cdc2* kinase utilizing addition of defined concentrations of baculovirus-produced clam cyclin A protein to RNase-treated Murray extracts. As shown in Fig. 3 A, control extracts carried out two consecutive cycles with a periodicity of 40–50 min that were absent in RNase-treated extracts to which lysates from wild-type-infected cells were added. These oscillations exhibited identical kinetics whether assayed in extracts or after immobilization on Sepharose beads covalently linked to p13<sup>SUC1</sup>. Fig. 3 B shows the effect of adding increasing amounts of cyclin A to an RNased extract. After a lag of 10 min, as little as 25 nM cyclin was able to restore one cycle of activation of p34<sup>cdc2</sup> kinase to approximately the same level as at metaphase in a control cycle

(Fig. 3 A). However, increasing amounts of cyclin A caused cycles with the same initial lag but with elevated maxima of p34<sup>cdc2</sup> kinase activity. The kinase activity maxima occurred at 45 min regardless of the amount of cyclin added, but initial rates of activation were higher as cyclin concentration increased. At high concentrations of cyclin ( $\geq 250$  nM), p34<sup>cdc2</sup> kinase activity became hyperactivated about eight-fold at the metaphase (45 min) point in the cycle, and it maintained high activity for several hours, behaving essentially like a CSF-arrested extract. Examination of nuclear morphology in such extracts confirmed that H1 kinase activity was a consistent measure of cell cycle stage (Table I). The inset in Fig. 3 B shows a linear relation between a 10-fold range of cyclin A protein added and the maximal level of p34<sup>cdc2</sup> kinase activation, suggesting a direct interaction between cyclin A and *cdc2* kinase. Consistent with the expected association of MPF subunits, experiments with [<sup>35</sup>S]methionine-labeled cyclin A from Sf9 cells showed that added cyclin could rapidly be recovered bound to *cdc2* kinase on p13<sup>SUC1</sup>-Sepharose beads (data not shown).

Also evident from these experiments was the decline in the



**Figure 4.** Clam cyclin A blocks degradation of *Xenopus* cyclins A, B1, and B2. Oscillating extracts were prepared and supplemented with [<sup>35</sup>S]Trans-label (ICN Radiochemicals, Irvine, CA) (1  $\mu$ Ci/ $\mu$ l extract before shifting to 23°C). Labeled cyclin/*cdc2* complexes were isolated using p13<sup>SUC1</sup>-Sepharose beads and analyzed by SDS-gel electrophoresis as described in Materials and Methods. Parallel samples were taken to determine the histone H1 activity of the isolated complexes. (Top) Untreated oscillating extracts (control). (Bottom) Oscillating extracts treated with lysate containing clam cyclin A (final concentration 250 nM).



**Figure 5.** Activation of *cdc2* kinase by cyclin A after exit from M phase. (A) Oscillating extracts were prepared and incubated as described in Materials and Methods. At 60 min after initiating the cycle (when the first M phase was completed), emetine was added to a final concentration of 100  $\mu$ M (thick arrow), and the extract was allowed to incubate further at 23°C. 35 min after emetine addition, the sample was divided into two aliquots, with one receiving clam cyclin A (thin arrow) (closed triangles, final concentration of

cyclin A, 25 nM). Emetine alone, open triangles. (B) CSF extracts were prepared as described in Materials and Methods. 0.2 mM CaCl<sub>2</sub> was added at 0 min and 80 min to all extracts. The control extract (○—○) was performed in the absence of emetine, and is the same extract in both B and C. 100  $\mu$ M emetine was added to the extracts at 0 min, then supplemented with either 250 nM cyclin A or wild-type Sf9 cell lysate (■—■) at 25 min (thin arrow), and the activity of *cdc2* kinase monitored by H1 kinase activity. (C) CSF extract was treated with calcium at 0 min and allowed to progress until arrest at first mitotic metaphase was evident. 100  $\mu$ M emetine was added to the extracts along with 0.2 mM CaCl<sub>2</sub> (80 min, thick arrow), which caused release from mitotic metaphase arrest. The extracts were then supplemented with either 250 nM cyclin A (▲—▲) or wild-type infected Sf9 cell lysate (■—■) at 105 min (thin arrow) and the activity of *cdc2* kinase monitored.

rate of p34<sup>cdc2</sup> kinase deactivation at progressively higher concentrations of cyclin (Fig. 3 B). To determine if this represented effects on cyclin degradation, studies were carried out with [<sup>35</sup>S]methionine in Murray extracts to which unlabeled cyclin A had been added. As shown in Fig. 4, clam cyclin A addition delayed the degradation of both the A- and B-type endogenous *Xenopus* cyclins in Murray extracts. This supports the idea that the level of cyclin A determines the rate of exit from M phase (like cyclin B, Murray and Kirschner, 1989) but does not normally control the rate of entry into M phase.

These results suggested that cyclin A in the absence of protein synthesis was sufficient to stimulate a round of *cdc2* kinase activation as well as subsequent degradation of the cyclin and kinase inactivation. A similar conclusion relative to cyclin B has been made earlier from the work of Murray and Kirschner. However, in the preparation of Murray extracts, eggs are activated for 30 min before crushing (see Materials and Methods) raising the possibility that the extract contained some level of newly synthesized cyclin or some other protein. This level would have to be rather low since RNase treatment caused arrest of the cell cycle (Fig. 3) and okadaic acid addition to extracts made at this time does not cause pre-MPF activation (Felix et al., 1990). To assess the ability of cyclin A to act alone more rigorously, we investigated cyclin A action in extracts that had already completed one cycle of MPF activation and inactivation in vitro. Fig. 5 A shows that addition of emetine after one cycle in an oscillating Murray extract abolishes further activation of *cdc2* kinase, but cyclin A addition still causes activation of *cdc2* kinase to the metaphase level. Control experiments have confirmed that all newly synthesized cyclins bound to p13<sup>SUC 1</sup> beads are degraded in these extracts at exit from M phase (Fig. 4, control). Other experiments examined cyclin A action after release of an extract from a CSF arrest. Such extracts retain the metaphase arrest of the unfertilized egg and can be stimulated to degrade cyclin, exit meiosis II, and enter mitotic cell cycles by addition of calcium ion, the normal trigger occur-

ring intracellularly at fertilization (Lohka and Maller, 1985; Solomon et al., 1991). As shown in Fig. 5 B, cyclin A addition to emetine-treated CSF extracts that had exited from meiosis II was able to cause a marked and stable hyperactivation of *cdc2* kinase. CSF extracts treated with calcium normally arrest again at first mitotic metaphase (Fig. 5 C), but a second addition of calcium also causes release from first mitotic metaphase. As shown in Fig. 5 C, cyclin A was able to activate *cdc2* kinase after a second M phase in vitro, indicating cyclin A does not require cyclin B or any other newly synthesized protein to activate *cdc2* kinase after exit from meiosis or mitosis.

## Discussion

The results presented in this paper provide new insights into the role of cyclin A in the cell cycle and the mechanism of activation of p34<sup>cdc2</sup>. Although both cyclins could stimulate both *Xenopus* meiotic cell cycles in the absence of protein synthesis, functional cyclin A protein was five times more potent than cyclin B. Previous work has shown that mRNA for both cyclin A and B can also drive meiotic cycles, although no quantitative comparison was made, and it could not be determined if synthesis of other proteins was required (Swenson et al., 1986; Westendorf et al., 1989). It is not immediately obvious why cyclin A should be more potent than cyclin B. One possibility, proposed by Hunt (Minshull et al., 1990), is that cyclin A/*cdc2* complexes activate cyclin B/*cdc2* complexes. This idea is made somewhat attractive by the fact that cyclin B/*cdc2* complexes preexist in the resting oocyte as "pre-MPF", whereas cyclin A is synthesized only after progesterone treatment (Gautier and Maller, 1991). Another possibility is that the oocyte maintains a mechanism to inactivate B-type "pre-MPF". Potentially, some of the injected cyclin B could be diverted to the inactive "pre-MPF" pool. Since cyclin A is absent in oocytes containing pre-MPF (Minshull et al., 1990), no inactivation mechanism for cyclin A might be

present, resulting in an apparent greater potency. Finally, it is conceivable cyclin B requires modification (phosphorylation, association with other components, etc.) before it can act.

Because both cyclin A and B proteins induce maturation after prolonged incubation in cycloheximide, it is evident both cyclins act downstream or independently of the *mos<sup>sc</sup>* proto-oncogene product, whose synthesis is required for meiosis in *Xenopus* (Sagata et al., 1988, 1989a). We previously demonstrated that *Xenopus* cyclin B2 could be phosphorylated in vitro by the *mos<sup>sc</sup>* kinase (Roy et al., 1990). This may indicate cyclin protein acts downstream of the *mos<sup>sc</sup>* product rather than independently. This idea is also supported by the absence of a stable metaphase II activation of p34<sup>*cdc2*</sup> by cyclin in the absence of the *mos<sup>sc</sup>* kinase (Fig. 2 B). Theoretically, *mos* could be present in insect cells and act together with cyclin to stimulate meiotic cycles. This seems unlikely because the Sf9 cell lysates are greatly diluted due to the enormous over-expression of cyclin, reducing the concentration of any noncyclin protein to trace levels. In addition, the *mos* protooncogene has not been detected below vertebrate phyla (Vande Woude, G., personal communication), and wild-type Sf9 cell lysates, even at high concentration, did not display the CSF activity of *mos<sup>sc</sup>* in oocytes or oscillating Murray extracts. Moreover, Western blotting of concentrated Sf9 cell lysates with an antibody to a highly conserved domain of *mos* (residues 37–55 of mouse *v-mos*, Maxwell and Arlinghaus, 1985) did not show any detectable *mos* protein (Roy, L. M., and J. L. Maller, unpublished data).

The experiments with cyclin A in both Murray extracts and CSF extracts provide the first evidence that A-type cyclins alone can also drive mitotic cell cycles. The potency of cyclin A to restore HI kinase to normal metaphase levels (25 nM) is similar to that previously found with sea urchin cyclin B (Murray and Kirschner, 1989; Solomon et al., 1991). But cyclin A was also able to hyperactive p34<sup>*cdc2*</sup> kinase, mostly likely by recruitment of additional monomeric *cdc2* kinase molecules into a complex, as judged by the ability to rapidly recover added cyclins on p13<sup>*SUC1*</sup>-Sepharose beads. At the highest concentration of cyclin A added, *cdc2* kinase appeared to reach a stably activated state that caused a metaphase arrest (Table I) and cyclin stabilization (Figs. 3 B and 5 B). This level of kinase activity is about 8–10× greater than that seen in a normal metaphase. Since newly added cyclin could be recovered bound to *cdc2* kinase on p13<sup>*SUC1*</sup> beads, the increase is due to an increased number of cyclin/*cdc2* complexes. This suggests ~90% of the *cdc2* in a control M phase extract is not normally activated. This idea is consistent with the recent work of Solomon et al. (1991) using cyclin B addition to egg extracts. In addition, gel filtration of *Xenopus* egg extracts coupled with Western blotting of *cdc2* shows 80–90% of the protein migrates with a monomer molecular weight, but all kinase activity is associated with a minor fraction eluting with other components at *M<sub>r</sub>* = 170,000 (Gabielli, B., and J. Maller, in preparation).

That cyclin A could prevent degradation of both *Xenopus* B-type cyclins suggests that the same protease acts on both types of cyclin, or alternatively, the same type of modification (phosphorylation?, ubiquitination?, etcetera) is required for degradation of both cyclins, and clam cyclin A competes for this reaction. In this connection Glotzer et al. (1991) have recently reported that a protein A–sea urchin cyclin B hybrid

molecule becomes multi-ubiquitinated at M phase in *Xenopus* egg extracts, suggesting the possibility that the ubiquitin conjugating system can be easily saturated. The rate of deactivation of *cdc2* kinase was progressively decreased as cyclin concentration increased, providing evidence that degradation of cyclin A is necessary to exit mitosis and may be the rate-limiting step for this cell cycle transition. Previous work with sea urchin cyclin B (Murray et al., 1989) indicates its degradation is also required, suggesting either cyclin is sufficient to maintain metaphase arrest.

When cyclin A was added to egg extracts, there was a consistent 10-min lag before kinase activation was evident. This lag was independent of cyclin concentration added, suggesting it was reflective of an event involved in altering the rate of *cdc2* kinase activation. Recent studies that appeared after submission of this paper suggest that a similar lag seen with cyclin B addition to egg extracts reflects tyrosine and threonine phosphorylation and dephosphorylation events on *cdc2* kinase (Solomon et al., 1991). Other studies show that the *cdc25* gene product causes tyrosine and threonine dephosphorylation of *cdc2* in egg extracts and reduces the lag seen with saturating concentrations of cyclin B (Kumagai and Dunphy, 1991). Apparently cyclin is only rate-limiting for *cdc2* kinase activation if its level is below that needed to form adequate amounts of MPF, a situation probably not seen *in vivo*. Therefore, it seems possible that the cyclin-independent lag in activation seen here with cyclin A may also reflect covalent modification of *cdc2* and the action of *cdc25* to induce tyrosine dephosphorylation of *cdc2* kinase. However, Ferrell et al. (1991) found tyrosine phosphorylation of *Xenopus cdc2* did not occur in mitoses 2–12, but a 10-min lag was still observed upon cyclin A addition to extracts that had already exited from first mitosis in vitro (Fig. 5 C).

Although our experiments and those reported by Solomon et al. (1991) have added cyclin at a single time point late in interphase, it may be useful in future work to examine other times of addition in the interest of finding differences in the properties of A- and B-type cyclin/*cdc2* complexes. Recently Hunt and co-workers (Minshull et al., 1990) found in time course studies of Murray extracts that cyclin A/*cdc2* complexes became activated shortly after cyclin synthesis, whereas B<sub>1</sub> and B<sub>2</sub> cyclin complexes accumulated in an inactive form and only became activated near the interphase/M phase boundary. This difference in the timing of A and B *cdc2* complexes may reflect a fundamental difference in the roles of the two cyclins, which so far have been largely indistinguishable by all available functional measurements. Further experiments with baculovirus-produced cyclin A and B should prove useful for studies comparing A- and B-type cyclins.

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