Activation of the *Xenopus* cyclin degradation machinery by full-length cyclin A

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**SUMMARY**

The entry into mitosis is dependent on the activation of mitotic forms of cdc2 kinase. In many cell types, cyclin A-associated kinase activity peaks just prior to that of cyclin B, although the precise role of cyclin A-associated kinase in the entry into mitosis is still unclear. Previous work has suggested that while cyclin B is capable of triggering cyclin destruction in *Xenopus* cell-free systems, cyclin A-associated kinase is not able to support this function. Here we have expressed a full-length human cyclin A in *Escherichia coli* and purified the protein to homogeneity by virtue of an N-terminal histidine tag. We have found that when added to *Xenopus* cell-free extracts free of cyclin B and incapable of protein synthesis, the temporal pattern of cyclin A-associated cdc2 kinase activity showed distinct differences that were dependent on the concentration of cyclin A added. When cyclin A was added to a concentration that generated levels of cdc2 kinase activity capable of inducing nuclear envelope breakdown, the histone H1 kinase activity profile was bi-phasic, consisting of an activation phase followed by an inactivation phase. Inactivation was found to be due to cyclin destruction, which was prevented by mos protein. Cyclin destruction was followed by nuclear reassembly and an additional round of DNA replication, indicating that there is no protein synthesis requirement for DNA replication in this embryonic system. It has been suggested that the evolutionary recruitment of cyclin A into an S phase function may have necessitated the loss of an original mitotic ability to activate the cyclin destruction pathway. The results presented here indicate that cyclin A has not lost the ability to activate its own destruction and that cyclin A-mediated activation of the cyclin destruction pathway permitted destruction of cyclin B1 as well as cyclin A, indicating that there are not distinct cyclin A and cyclin B destruction pathways. Thus the ordered progression of the cell cycle requires the careful titration of cyclin A concentration in order to avoid activation of the cyclin destruction pathway before sufficient active cyclin B/cdc2 kinase has accumulated.

Key words: Cell cycle, Cyclin destruction, DNA replication, *Xenopus*

**INTRODUCTION**

Cyclin proteins were first identified in the eggs of marine invertebrates by their characteristic gradual accumulation during interphase and abrupt disappearance at mitosis (Evans et al., 1983). A family of related proteins has since been identified in many diverse species (Hunter and Pines, 1994) and their common primary function is the activation of the protein kinase subunit of members of the cdk family (Nurse, 1990). Many of these enzymes are key regulators of cell cycle progression, with discrete cell cycle transitions being governed by particular cyclin/cdk complexes.

The transition from interphase to mitosis is dependent upon the activation of the p34<sup>cdc2</sup> protein kinase subunit and a B-type cyclin (Dunphy et al., 1988; Gautier et al., 1990; Draetta et al., 1989; Labbe et al., 1989; Lohka et al., 1988). Evidence from micro-injection experiments with human cells that have completed S phase, suggests a possible role for a cyclin A-associated kinase in the progression from G2 to mitosis (Pagano et al., 1992). Indeed, in *Xenopus* egg extracts, cyclin A-dependent protein kinase activity has been shown to peak earlier than cyclin B/cdc2, suggesting that cyclin A may act upstream of cyclin B (Minshull et al., 1992). Consistent with this idea, mutant *Drosophila* embryos lacking cyclin A arrest in G2 (Lehner and O’Farrell, 1990). Cyclin A is known to be capable of inducing some events of mitosis at least in vitro (Roy et al., 1991). However, the precise role of cyclin A in the control of cell cycle progression is unclear. Micro-injection of anti-cyclin A antibodies in human cells prior to S-phase also blocks DNA replication (Pagano et al., 1992). In *Xenopus*, cyclin A has been implicated in the checkpoint that couples the completion of S-phase to the initiation of mitosis (Walker and Maller, 1991). Elevated levels of cyclin A-associated kinase activity have been reported in apoptotic human cell lines, suggesting that cyclin A may play a role in this process also (Meinkrantz et al., 1994).

Exit from mitosis requires the inactivation of the cyclin B/cdc2 kinase, which is brought about by the ubiquitin-mediated proteolytic degradation of the cyclin subunit (Sudak, 1995; Murray, 1995). A conserved region in
mitotic cyclins, termed the destruction box, is essential for proteolysis of the cyclin polypeptide (Murray and Kirschner, 1989; Glotzer et al., 1991; Lorca et al., 1992a; Gallant and Nigg, 1992). While the degradation of both cyclins A and B depend upon this conserved sequence, the A type cyclin is destroyed slightly earlier in the cell cycle than cyclin B (Luca and Ruderman, 1989; Minshull et al., 1990; Whitfield et al., 1990; Hunt et al., 1992). In addition to this, disruption of spindle assembly using colchicine markedly inhibits cyclin B destruction although similar treatment has no effect on cyclin A (Whitfield et al., 1990; Hunt et al., 1992).

Up-regulation of the cyclin degradation pathway at the end of mitosis is brought about by active p34cdc2. One target of p34cdc2 in this process is likely to be the cyclin selective ubiquitin protein ligase E3-C, which is inactive in extracts derived from interphase cells but active in mitotic extracts (Hershko et al., 1994). It is not clear, however, whether the abrupt onset of cyclin degradation is solely due to the activation of a ubiquitin protein ligase, or also involves activation of the substrate for proteolysis. The kinetics of E3-C activation suggest the existence of one or more intermediates between p34cdc2 and E3-C (Sudakin et al., 1995).

Recently, it has been reported that addition of either cyclin B or cyclin B/cdc2 to interphase egg extracts triggered the degradation machinery while cyclin A did not, though it formed an active kinase complex. Indeed, the cyclin A protein used seemed to delay the destruction of both A and B type cyclins (Luca et al., 1991; Lorca et al., 1992b). This led to the implication that some element of cyclin B structure confers the substrate for proteolysis. The RB-related protein p107, with which it associates during S-phase, is required the loss of its cyclin degradation function, thus avoiding the possibility that cyclin A kinases active in S-phase could be degraded (Humphries et al., 1990; Hunt et al., 1992). In addition to this, disruption of nuclear assembly morphology was monitored by removing 2 μl of extract, diluting with an equal volume of Fix/Stain solution (200 mM sucrose, 5 mM MgCl2, 7.5% formaldehyde, 10 μg/ml Hoechst 33258) and subjecting the sample to phase-contrast and fluorescence microscopy using an Olympus BH epifluorescence microscope.

### Nuclear assembly assays

These were done by adding demembranated sperm chromatin, prepared as described (Smythe and Newport, 1991) to interphase extracts containing an ATP-regenerating system. Progress of nuclear assembly morphology was monitored by removing 2 μl of extract, diluting with an equal volume of Fix/Stain solution (200 mM sucrose, 5 mM MgCl2, 7.5% formaldehyde, 10 μg/ml Hoechst 33258) and subjecting the sample to phase-contrast and fluorescence microscopy using an Olympus BH epifluorescence microscope.

### Purification of human cyclin A protein over-expressed in E. coli

A 1.5 kb NcoI/BamHI fragment containing the total human cyclin A coding sequence (a gift from J. Pines, Wellcome/CRC Institute) was ligated at the 5’ end onto NcoI/EcoRI cut pSL1180 (Pharmacia), which had been previously cut with SmaI and Bsu36I, endfilled and ligated. The linear pSL1180-cyclin A ligation product was size selected by agarose gel electrophoresis and purified using GeneClean II (Stratech Ltd) The Ndel site in pSL1180 immediately upstream of the NcoI site, was cut and the resultant NdeI/BamHI fragment containing the cyclin A coding sequence cloned into NdeI/BamHI-digested pET 15b (Novagen) to yield pCJ1. DNA sequencing (Pharmacia T7 kit) confirmed the integrity of the coding sequence.

The construct was used to transform the E. coli strain BL21 (DE3) pLysS and a mid log phase culture of the resultant clone induced with 0.5 mM IPTG (Gibco-BRL) and grown at 28°C overnight. Cells were pelleted by centrifugation and resuspended in Lysis buffer A (500 mM NaCl, 100 mM KCl plus 1 mM benzamidine and 0.1 mM PMSF, 2 mM K-Hepes, pH 7.0) before treatment with lysozyme (at 0.2 μg/ml) for 10 minutes until viscous. Cells were broken and DNA was sheared by sonication using a microtip at maximum intensity for 5 minutes. The lysate was spun in a Beckman JA-18 rotor at 15,000 rpm, at 4°C for 15 minutes and the supernatant incubated with 3 ml of packed nickel-agarose beads (Qiagen) with mixing for 30 minutes at 4°C. The resin was washed with a total of 100 volumes of 20 mM K-Hepes, pH 7.0, 500 mM NaCl, 100 mM KCl, 0.5% Triton X-100, 0.5% Tween-20 followed by 10 volumes of 20 mM K-Hepes, pH 7.0, before elution using 20 mM K-Hepes, pH 7.0, 150 mM imidazole. The eluate containing cyclin A was concentrated to 2.5 μg/ml and buffer exchanged into XB (10 mM K-Hepes, pH 7.7, 50 mM KCl, 2 mM MgCl2) using a Centricon 30 (Amicon Ltd) before freezing in liquid nitrogen and storing at −70°C.

### In vitro transcription/translation reactions

The pET15b vector containing human full-length cyclin A coding and a pGEM vector containing full-length coding for Xenopus cyclin B1 were used as templates in separate coupled transcription/translation reactions (Promega TNT system) to produce [35S]methionine-labelled proteins. The reaction was as described in the manufacturer’s directions, briefly: 1 μg DNA template was added to the reticulocyte lysate.

### MATERIALS AND METHODS

#### Egg extracts

Interphase egg extracts were prepared from unfertilised *Xenopus laevis* eggs as follows. Eggs were dejellied in 2% cysteine and transferred to soak for 15 minutes in 0.2X MMR (20 mM NaCl, 0.4 mM KCl, 0.2 mM MgSO4, 0.4 mM CaCl2, 0.02 mM EDTA, 1 mM K-Hepes, pH 7.7) + 100 μM cycloheximide. Eggs were activated by electrical stimulation (two 3-second 12 V AC pulses separated by a 5-second gap) and transferred to soak in egg lysis buffer (250 mM sucrose, 2.5 mM MgCl2, 50 mM KCl, 20 μg/ml cycloheximide, 1 mM dithiothreitol, 10 mM K-Hepes, pH 7.7) for an additional 20 minutes. Eggs were transferred to centrifuge tubes, packed and lysed by centrifugation as described (Smythe and Newport, 1991). The cytoplasmic fraction was removed and an ATP-regenerating system added (1/100th volume each of 0.5 μg/ml creatine kinase in water, 0.2 M ATP in 10 mM K-Hepes, pH 7.7, plus 1/10 volume of 0.2 M phosphocreatine in potassium phosphate buffer, pH 7.0).

Release of the extract into interphase was achieved by the addition of 0.4 mM CaCl2. The resulting extracts were treated with 50 μg/ml cycloheximide to prevent any endogenous cyclin synthesis.

#### In vitro transcription/translation reactions

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Western blotting and quantification of auto-radiographic images
The cyclin A preparation was resolved by SDS-PAGE on a 10% gel and transferred by capillary electrophoresis (Bio-Rad Ltd) for 250 volt hours at 4°C to 0.45 µm nitrocellulose (Schleicher & Schuell) in blotting buffer: 25 mM Tris, 192 mM glycine, 20% methanol. Blots were incubated at room temperature for 2 hours in TBST (150 mM NaCl, 0.1% Tween-20, 20 mM Tris-HCl, pH 8.0) plus 5% foetal calf serum, before incubation with anti-human cyclin A mouse monoclonal primary antibody (PharMingen Ltd) in the same buffer for 1 hour. Blots were washed three times for 5 minutes each in TBST before incubation with anti-mouse secondary antibody conjugated to horseradish peroxidase (Vectastain), washed 3 times again and bands were detected by enhanced chemiluminescence (ECL, Amersham). The intensities of images on film were quantified using a Molecular Dynamics laser scanning densitometer.

Protein kinase assays
Cyclin-dependent kinase activity in interphase egg extracts was measured essentially as described (Smythe and Newport, 1991). MAP kinase was assayed in an identical way except that the reaction mixture contained 10 µg myelin basic protein as substrate.

DNA replication assays
DNA replication was measured as described (Smythe and Newport, 1991). Samples (4 µl) of extract were removed to a separate tube containing 1 µCi of [α-32P]dATP and incubated for 20 minutes at room temperature. The reaction was stopped by the addition of 10 µl replication sample buffer (8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% SDS, 0.2% Bromophenol blue, 80 mM Tris-HCl, pH 8.0). Protein in each sample was digested with proteinase K at a final concentration of 1 µg/ml for 2 hours at room temperature. Samples were then resolved by electrophoresis on an 8% agarose gel, which was dried and exposed to pre-flashed X-ray film for autoradiography.

RESULTS
Kinetics of cyclin A-dependent histone H1 kinase activity in interphase extracts
A cDNA encoding full-length human cyclin A was sub-cloned into a modified form of pET 15b containing coding sequence for an N-terminal hexahistidine tag. Following transformation of E. coli strain BL21 (DE3) pLysS, the protein was expressed (as described in Methods) and purified to homogeneity by Ni-NTA affinity chromatography. This yielded a single homogeneous polypeptide of the expected molecular mass (55 kDa) as judged by SDS-PAGE (Fig. 1, lane a). Western blotting with a mouse monoclonal anti-human cyclin A antibody confirmed the identity of the purified protein (Fig. 1, lane b). Expression of the human cyclin A construct in reticulocyte lysates yielded a single [35S]methionine-labelled band of identical molecular mass (Fig. 1, lane c).

We wished to determine whether the human cyclin A was functionally active in a Xenopus cell-free system. Therefore, the protein was introduced into extracts prepared from Xenopus eggs that had been activated by electric shock and maintained for 20 minutes after activation in the presence of cycloheximide before lysis (Lorca et al., 1992b). This procedure results in the temporary activation of the cyclin destruction pathway, which results in complete degradation of the endogenous cyclins. The presence of cycloheximide prevents the reaccumulation of any endogenous cyclins. Extracts prepared in this way have been shown to contain no cyclin B (Lorca et al., 1992b; Clarke et al., 1992; C. Smythe, unpublished) and cannot carry out any further protein synthesis (not shown). Consistent with this, we found that addition of protein phosphatase inhibitor microcystin (which induces the activation of latent cyclin B/cdc2; Yamashita et al., 1990) had no effect on the morphology of nuclei assembled in these extracts (data not shown).

Addition of 300 nM purified cyclin A to interphase egg extracts resulted in activation of histone H1 kinase activity to a stable level just below the threshold required by cyclin B/cdc2 (~15 pmol phosphate/minute per µl) to drive entry into mitosis (Fig. 2A). Full activation by this concentration of cyclin A was observed after 70 minutes of incubation in the extract. Surprisingly, the addition of higher levels (600 nM) caused a transient peak of kinase activity, which was maximal at approximately 35 minutes and returned to basal levels by approximately 80 minutes (Fig. 2A).

Mechanism of cyclin A-dependent kinase inactivation
In order to determine the mechanism of inactivation of cyclin A-dependent kinase in this system, we examined the stability of the cyclin subunit using [35S]methionine-labelled cyclin A. Previous work has shown that the specific proteolytic degradation of mitotic cyclins may be assayed by sampling extract containing 35S-labelled cyclin at various times and analysing the 35S-labelled products by SDS-PAGE (Felix et al., 1990; Lorca et al., 1992a,b; Stewart et al., 1994). We found that at a concentration of 300 nM cyclin A, the labelled band remained completely stable throughout the course of the experiment (>80 minutes, Fig. 2B). In contrast, the addition of 600 nM cyclin A...
to the extract resulted in a progressive loss of the $[^{35}\text{S}]$methionine-labelled band. Significant destruction of the cyclin A coincided with the peak of histone H1 kinase activity and was complete by 80 minutes (Fig. 2A,B and C). These data show that the concentration-dependent inactivation of the histone H1 kinase was due to destruction of the cyclin subunit of the protein kinase complex. Interestingly, when we added cyclin A to the extract at 4.5 $\mu$M, a concentration above that required to saturate the endogenous supply of cdc2 protein, we found that this resulted in very high levels of kinase activity that remained stable for the duration of the experiment (Fig. 3).

**Fig. 2.** Analysis of cyclin A-dependent kinase activity and cyclin A stability in egg extracts. (A) The indicated concentrations of purified human cyclin A was added to interphase extracts prepared as described in Materials and Methods. Samples were withdrawn at 10 minute intervals for determination of cyclin A-dependent histone H1 kinase activity. (B) $[^{35}\text{S}]$methionine-labelled cyclin A was produced in a reticulocyte lysate and a trace amount of the radio-labelled protein was added to interphase extract immediately prior to the addition of either 300 nM or 600 nM unlabelled cyclin A purified from bacteria. Samples were removed at indicated times and subjected to SDS-PAGE and autoradiography. (C) The intensity of the labelled bands in B was determined by laser-scanning densitometry.

**Fig. 3.** Saturation of cyclin destruction with high concentrations of cyclin A. The indicated concentrations of purified human cyclin A were added to interphase extracts prepared as described in Materials and Methods. Samples were withdrawn at 10 minute intervals for determination of cyclin A-dependent histone H1 kinase activity.

**Nuclear envelope breakdown and reassembly in interphase extracts containing cyclin A**

Elevated levels of cyclin A have been reported to correlate with the onset of apoptosis (Meikrantz et al., 1994) and *Xenopus* egg extracts have been reported to be a model system for the study of apoptosis in vitro (Newmeyer et al., 1994). We therefore determined whether the destruction of cyclin A observed in Fig. 2 was a consequence of the initiation of apoptotic-like events by cyclin A. Purified cyclin A was added to egg extracts containing demembranated sperm chromatin, to a final concentration of 300 or 600 nM. Aliquots of the extract were removed at the indicated times and nuclear envelope and DNA morphology were examined by light and fluorescence microscopy. Nuclei in extracts containing 300 nM cyclin A (Fig. 4, upper panels) remained intact and continued to enlarge throughout the course of the experiment.

**Fig. 4.** Entry into and exit from mitosis may be driven by cyclin A. Interphase extracts were incubated at room temperature in the presence of demembranated sperm chromatin to initiate nuclear assembly. Aliquots of the extract were immediately treated with 300 or 600 nM cyclin A and the nuclear morphology was monitored at the indicated times by fluorescence (upper panels) using Hoechst 33258 to detect DNA, and phase-contrast (lower panels) microscopy, as described in Materials and Methods. Bar, 5 $\mu$m.
Cyclin A-mediated cyclin destruction

of the experiment (up to 2.5 hours). The morphology of nuclei exposed to 300 nM cyclin A was no different to controls to which no cyclin A was added (not shown; Hutchison et al., 1988). We found that the addition of 600 nM cyclin A caused nuclear envelope breakdown (NEBD) and chromosome condensation 30 minutes after addition (Fig. 4, lower panels), coinciding with the peak of cyclin A-dependent histone H1 kinase activity (cf Fig. 2A and data not shown). However, following destruction of the cyclin A protein, a second round of nuclear envelope assembly and chromosome decondensation was observed (Fig. 4, lower panels). We also examined the ability of nuclei that reformed after cyclin A destruction to undergo an additional round of DNA replication. Extracts containing demembranated sperm chromatin were incubated at room temperature and, at the indicated times, aliquots were removed and the extent of ongoing DNA synthesis measured by the incorporation of label from $[^{32}\text{P}]$dATP into DNA. In this experiment, cyclin A was added 70 minutes after initiation of nuclear assembly. (A) Effect on DNA replication of addition of 300 nM cyclin A. (B) Effect on DNA replication of addition of 600 nM cyclin A.

mos prevents but does not inhibit the cyclin A-mediated cyclin destruction pathway

We wished to establish that the destruction of cyclin A that occurred when this protein was introduced into an extract was due to activation of the specific cyclin destruction pathway and not the result of some non-specific destruction in the egg extract. To do this, we utilised the fact that p39$^{\text{mos}}$, the protein product of the mos proto-oncogene, is a component of cytosstatic factor, which arrests cells in metaphase, by specifically blocking the mitotic destruction of cyclins (Sagata et al., 1989; Yew et al., 1992). We therefore added malE-mos fusion protein, expressed in and purified from E. coli (Nebreda and Hunt, 1993), to Xenopus extracts, either 30 minutes prior to, or at the same time as, the addition of 600 nM cyclin A. We determined the amount of mos protein that resulted in the activation of MAP kinase to the extent observed in a cytostatic factor-arrested (CSF) extract (Murray, 1991). When this level of malE-mos was added to extracts 30 minutes prior to the addition of cyclin A, the inactivation of the cyclin A-dependent histone H1 kinase activity was prevented, suggesting that the destruction of the cyclin A was blocked under these conditions (Fig. 6). The prevention of cyclin degradation by malE-mos
was not due to any reduction in the level of cyclin A-dependent histone H1 kinase activity, which peaked at the same level in the presence and absence of the mos protein (Fig. 6A). Nor was the lack of disappearance of histone H1 kinase activity due to the activation of additional histone kinases in the extract by malE-mos, as no detectable histone kinase was observed when malE-mos alone was added to extracts (data not shown). Interestingly, addition of the malE-mos protein to extracts at the same time as cyclin A, did not result in stabilisation of the histone H1 kinase activity (Fig. 6C). This was not due to any reduction in the efficiency with which malE-mos protein activated the MAP kinase pathway as the level of MBP kinase activity that resulted from the addition of malE-mos was the same whether the mos was added at the same time or prior to cyclin A addition (data not shown). These data show that mos can act in a cell-free system to block inactivation of a mitotic cyclin-dependent kinase, but is incapable of blocking inactivation once the latter has been initiated.

**Activation of cyclin B destruction with cyclin A**

Active cyclin B/cdc2 complexes have been shown to induce the destruction of both cyclin A and cyclin B (Lorca et al., 1992b). The results presented above indicate that cyclin A can activate the destruction of both cyclin A and cyclin B (Lorca et al., 1992b). The results presented above indicate that cyclin A can activate the destruction of cyclin B. These data strongly support the notion that a single cyclin destruction machine acts on both A and B type cyclins at mitosis.

We found that there was a strong threshold effect in the ability of cyclin A to activate the cyclin destruction pathway. In addition there was an absolute correlation between the concentration of cyclin A required to induce cyclin destruction and nuclear envelope breakdown, such that the concentration of cyclin A required to induce cyclin destruction was indistinguishable from that required to initiate nuclear envelope breakdown. Concentrations of cyclin A at 300 nM or below never induced cyclin degradation or NEBD, although at this concentration the cyclin was relatively stable and kinase activity remained elevated for extended periods (Fig. 2A and data not shown). Concentrations of cyclin A intermediate between 300 nM and 600 nM were capable of inducing NEBD and cyclin destruction, but this tended to vary slightly from extract to extract. We therefore utilised 600 nM cyclin A, which always induced NEBD and always induced cyclin destruction, in the experiments described here.

These results indicate that entry into mitosis requires a threshold concentration of a cyclin/cdk complex. In the experiments described above, the active complex is likely to be cyclin A/cdc2, because in *Xenopus* eggs, unlike somatic cells, both human and *Xenopus* cyclin A have been shown to bind cdc2 exclusively (Devault et al., 1992; Minshull et al., 1990). The requirement for a threshold concentration of cyclin A/cdc2 may reflect the presence of a kinase inhibitor that must be titrated out in order to effect the phosphorylation of target proteins necessary for mitosis. A number of small protein heat-stable inhibitors have been described that act as brakes on cell cycle progression (Elledge and Harper, 1994), although none has yet been described for the G2/M transition. Alternatively, the threshold may result from the constitutive activities of the protein phosphatases that act to oppose cdks (Ferrigno et al., 1993). If true, this would indicate that the ability of a cyclin/cdk to promote mitosis is a consequence of successfully overwhelming a futile cycle involving the continuous phosphorylation and dephosphorylation of target sites on proteins. Future work will determine whether the threshold is the con-
sequence of a cdk inhibitor or kinase/phosphatase futile cycle creates the threshold for the G2 to M transition.

The protein product of the mos oncogene is a component of cytostatic factor, which arrests cells in metaphase by stabilising mitotic cyclin/cdk complexes (Sagata et al., 1989). Recent work has shown that mos protein activates the MAP kinase pathway, by phosphorylating and activating the upstream activator of MAP kinase, MEK (Posada et al., 1993), and that stabilisation of mitotic cyclins occurs via activation of MAP kinase (Minshull et al., 1994). However, little is known about the signalling mechanism by which activation of MAP kinase achieves stabilisation of cyclins. We found that the addition of recombinant mos protein into *Xenopus* interphase extracts activated MAP kinase as reported (Nebreda and Hunt, 1993), as judged by MBP kinase activity. We have found that activation of MAPK by mos protein prevented the inactivation of cyclin A/cdc2, presumably by preventing cyclin A-mediated cyclin destruction, thus recreating a CSF-like state in this cell-free system (Fig. 6). This observation supports the view that, in the in vitro system reported here, the destruction of cyclin A is occurring via the mechanism that operates at the end of M phase. Interestingly, we found that although mos protein clearly prevented the initiation of cyclin destruction, mos was not able to inactivate the cyclin destruction pathway once started by cyclin A, as addition of mos protein to the extract at the same time as cyclin A did not block kinase inactivation, and the cdc2 activity ultimately returned to base line levels (Fig. 6C). These results suggest that the outcome following activation of MAPK and cdc2 pathways may be critically dependent on the relative timing of activation of these two pathways, and may explain why cells that contain both active cyclin/cdc2 complexes and MAPK do not necessarily arrest in metaphase (Tamemoto et al., 1992).

In direct contrast to the results presented here, previous work has suggested that cyclin A/cdc2, unlike cyclin B/cdc2, is unable to activate the destruction pathway, as the expression of human cyclin A in a *Xenopus* cell-free system (Lorca et al., 1992b), and N-terminally truncated forms of clam cyclin A in a clam cell-free system (Luca and Ruderman, 1989) failed to induce, and even delayed cyclin degradation. We do not know the reason why our results apparently differ from those of Lorca et al. (1992b). However, we have found that a concentration window exists in which cyclin A-mediated cyclin destruction may be observed. At very high concentrations of cyclin A, the cyclin destruction pathway is inhibited (Fig. 3; Lorca et al., 1992b). This may be due to either the presence or the production of sufficient levels of a non-productive intermediate that blocks the destruction pathway. In addition, we have found that the levels of cyclin A-associated histone H1 kinase required to induce NEBD and cyclin destruction is significantly higher than that observed for cyclin B/cdc2. Cyclin A concentrations that do not generate this level of histone H1 kinase never induce cyclin destruction in the extract (Fig. 4).

As Lorca et al. utilised cyclin A/cdc2, which generated levels of kinase activities similar to cyclin B/cdc2 during meiotic M-phase, without establishing whether they were sufficient for NEBD in vitro, it is possible that these levels were too low to initiate destruction (Lorca et al., 1992b).

In *Xenopus*, the cdc2 catalytic subunit is present throughout the cell cycle at a concentration of 500 to 600 nM (Kobayashi et al., 1994) but it is inactive as a protein kinase in the absence of a cyclin partner. Experiments using recombinant cyclin B have demonstrated that a threshold activity of kinase is required to initiate mitosis and typically 100-200 nM cyclin B is required for this (Devault et al., 1992; Lorca et al., 1992a; Solomon et al., 1990). We have found that 2- to 3-fold higher levels of full-length cyclin A were required to trigger nuclear envelope disassembly and cyclin destruction. Consistent with this observation, we also found that the specific activity of the full-length cyclin A-activated cdc2 kinase was appropriately lower than that reported by others using various truncated recombinant cyclin A constructs (Clarke et al., 1992; Devault et al., 1992). This requirement for higher levels of full-length cyclin A may be the consequence of a high proportion of incorrectly folded protein, a higher association constant for cdc2 binding, or a lower specific activity of the resulting full-length cyclin/cdc2 complex towards mitotic substrates. We have consistently observed higher specific activities in complexes formed using N-terminally truncated cyclin A (Jones and Smythe, unpublished observations). Many investigations of cyclin A function have involved the use of N-terminally truncated versions of the cyclin protein. This has been in part due to the fact that such constructs facilitate recombinant expression, retaining biological activity and solubility at high concentration. The expression of full-length cyclin A has been more problematic, although reliable expression has been obtained in reticulocyte lysates. We have been unable to determine whether other A-type cyclins can trigger cyclin destruction using reticulocyte lysates, because the final cyclin concentration is insufficient to generate mitotic levels of cdc2 activity.

Hunt and colleagues (Kobayashi et al., 1994) have estimated the concentrations of cyclins present in eggs arrested in metaphase by immunoblotting egg extracts compared to purified expressed cyclin proteins. In contrast to the concentrations discussed above, these results suggest that the metaphase concentration of cyclin B is about 10 nM and of cyclin A about 10-fold less. These data suggest either that the specific activities of recombinant cyclin/cdc2 complexes are far lower than the native proteins, or that epitope suppression during immunoblotting results in an underestimate of the cyclin concentration in vivo.

It has been suggested (Lorca et al., 1992b) that activation of the cyclin degradation pathway by cyclin A must occur via the cyclin A-mediated activation of cyclin B/cdc2. A number of lines of evidence indicate that the potentiation of the cyclin destruction pathway reported here is triggered by cyclin A directly and not indirectly via the cyclin A-mediated activation of cyclin B/cdc2 complexes present in the extract. Firstly, extracts were prepared in a manner that ensured that the levels of cyclin B were minimal, by pre-incubation of eggs in cycloheximide to block new protein synthesis, together with an electrical activation step to ensure synchronous exit from meiotic metaphase and thus destruction of endogenous cyclins. Secondly, consistent with the notion that cyclin B cannot be responsible, we found that the addition of 1 μM microcystin to the extracts, which results in the potentiation of cyclin B/cdc2 by eliminating the threshold and the lag associated with the activation of cyclin B/cdc2 in oscillating extracts (Solomon et al., 1990), did not potentiate mitosis in the interphase extracts used here, as judged by the criterion of nuclear envelope breakdown (data not shown). As the levels of cyclin/cdc2
kinase required to initiate the cyclin destruction pathway cannot be less than those required for nuclear envelope breakdown, these results clearly indicate that there is insufficient cyclin B/cdc2 present in these extracts to induce the cyclin destruction pathway.

In *Xenopus*, the role of cyclin A is far from clear. There is no requirement for de novo synthesis of cyclin A for DNA replication following exit from meiotic metaphase nor does it appear to be necessary for the G2 to M transition. In striking contrast to other organisms, ablation of cyclin A1 results in replication following exit from meiotic metaphase nor does it involve subthreshold levels of both kinases suggest that there is no synergy in activating the pathway, but further work is required on this issue. As we have found that the threshold amount of cyclin A required to induce cyclin degradation is similar to that required to induce cyclin A-mediated nuclear envelope breakdown, this may only happen in circumstances where cyclin A levels reach this amount. Although the levels of cyclin A have not been measured in cells in G2, one situation in which cyclin A concentrations might approach these levels would be in cells arrested in G2 as a consequence of DNA damage, wherein cyclin B/cdc2 activation and mitosis is blocked. It seems likely that mechanisms may exist for the regulation of cyclin A-associated kinase activity to prevent cyclin A-mediated mitosis under these circumstances.

Finally, initiation of DNA replication is controlled by at least two distinct entities. One of these, replication licensing factor (RLF), ensures that eukaryotic chromosomal DNA is replicated exactly once in each cell cycle (Blow, 1993; Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995; Yan and Newport, 1995). On exit from metaphase, RLF is generated, and licences DNA replication. RLF is inactivated as replication begins as a consequence of DNA damage, wherein cyclin B/cdc2 activation and mitosis is blocked. It seems likely that mechanisms may exist for the regulation of cyclin A-associated kinase activity to prevent cyclin A-mediated mitosis under these circumstances.

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