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Phosphorylation of *Xenopus* Cyclins B1 and B2 Is Not Required for Cell Cycle Transitions

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The *cdc2* kinase and B-type cyclins are known to be components of maturation- or M-phase-promoting factor (MPF). Phosphorylation of cyclin B has been reported previously and may regulate entry into and exit from mitosis and meiosis. To investigate the role of cyclin B phosphorylation, we replaced putative *cdc2* kinase phosphorylation sites in *Xenopus* cyclins B1 and B2 by using oligonucleotide site-directed mutagenesis. We found that Ser-90 of cyclin B2 and Ser-94 or Ser-96 of cyclin B1 are the main phosphorylation sites both in functional *Xenopus* egg extracts and after phosphorylation with purified MPF in vitro. Microtubule-associated protein (MAP) kinase from *Xenopus* eggs phosphorylated cyclin B1 significantly at Ser-94 or Ser-96, whereas it was largely inactive against cyclin B2. The substitutions that ablated phosphorylation at these sites, however, resulted in no functional differences between mutant and wild-type cyclin, as judged by the kinetics of M-phase degradation, induction of mitosis in egg extracts, or induction of oocyte maturation. These results indicate that the phosphorylation of *Xenopus* B-type cyclins by *cdc2* kinase or MAP kinase is not required for the hallmark functions of cyclin.

The onset of M-phase is regulated by a mechanism common to all eukaryotic cells. The key component regulating M-phase is called maturation- or M-phase-promoting factor (MPF). MPF is now known to be a complex of the *cdc2* kinase with B-type cyclins (for reviews, see references 21, 29, and 32). However, simple association of the two subunits is not sufficient for activation, as judged by the presence of pre-MPF cyclin/*cdc2* complexes in *Xenopus* oocytes (8) and the lag often seen between accumulation of cyclin to high levels and the activation of *cdc2* (24, 31). Additional post-translational modifications of MPF components are required. These modifications include phosphorylation (38) and dephosphorylation (4, 9, 11, 26) on tyrosine and possibly threonine residues of the *cdc2* kinase itself. Another possible mechanism is the phosphorylation of cyclin B. In *Xenopus* cells, there are two closely related B-type cyclins, designated B1 and B2 (23). Cyclin B has been reported to be phosphorylated in *Xenopus* oocytes and eggs (8) and in cultured human cells (31). Furthermore, the appearance of histone H1 kinase activity in cleaving sea urchin eggs has been shown to correlate closely with phosphorylation of the cyclin subunit of the H1 kinase complex (22). Since the *cdc2* kinase is complexed with both cyclin B1 and cyclin B2 and purified MPF is known to phosphorylate cyclin B2 in vitro (10), one kinase that phosphorylates cyclin B is probably the *cdc2* kinase itself, although other kinases such as the *Xenopus* c-*mos* product may also phosphorylate cyclin B2 (34).

In this investigation, we have studied the sites and the role of cyclin B phosphorylation by constructing mutants of *Xenopus* cyclins B1 and B2. We found that (i) Ser-90 of cyclin B2 and Ser-94 or Ser-96 of cyclin B1, the only serines which are followed by proline residues, are the main phosphorylation sites of these cyclins in *Xenopus* egg extracts; (ii) these sites are also the only ones phosphorylated by purified MPF in vitro; (iii) cyclin B1 can also be phosphorylated by *Xenopus* microtubule-associated protein (MAP) kinase with similar specificity; and (iv) the elimination of these phosphorylation sites does not affect cyclin degradation during exit from M-phase, induction of mitosis, or oocyte maturation.

MATERIALS AND METHODS

Site-directed mutagenesis of cyclin cDNAs. *Xenopus* cyclin B1 and B2 cDNAs (23) were kindly provided by Tim Hunt (ICRF Clare Hall Laboratories, United Kingdom) and Jeremy Minshull (University of California, San Francisco). Site-directed mutagenesis of cyclin B1 and B2 cDNAs was performed as described by Kunkel et al. (14). Normal cyclin B2 cDNA contained in pGEM1 was excised by *Eco*RI and cloned into the *Eco*RI sites of M13mp18. Cyclin B1 cDNA contained in pGEM1 (designated pGEMB1) was excised by *Eco*RI and *Bam*HI digestion and also cloned into the *Eco*RI and *Bam*HI sites of M13mp18. Synthetic deoxyoligonucleotides were chemically synthesized and used as mismatched primers to introduce Ser-to-Ala mutations or Thr-to-Val mutations by annealing with a single-stranded uracil residue-containing template of the *Xenopus* cyclin B2 noncoding strand or B1 coding strand (Fig. 1). The following oligonucleotides were used: B2 Ala 90, 5′-GCCCCCAAAGTGCCGGCCCCTGTGCCGATG-3′; B2 Val 224, 5′-TATGAAGAGATGTACGTACCAGAGGTTGCAGAC-3′; B1 Ala 94 Ala 96, 5′-CAGATGTTTCCATTGGGGCTGGCGCGCTGGGTTCAACCTGAG-3′; B1 Val 306, 5′-CTGCTATTTGGGAAGGCACGAAGTGTACCATATCATAATC-3′; and B1 Val 328, 5′-TGATGGAGTGTTGGGACCCAGTCACCTGCATT-3′. The mutated bases are underlined. Putative mutants were first identified by *Nae*I (B2 Ala 90), *Sna*B1 (B2 Val 224), *Bss*HII (B1 Ala 94 Ala 96), *Dra*III (B1 Val 306), and *Eco*O109I (B1 Val 328) digestion, the site of which is introduced artificially in each oligonucleotide. All sequences were confirmed by direct DNA sequence analysis. In the case of B1 Val 306 and B1 Val 328, both oligonucleotides were used at the same time as primers to produce double mutants. The mutated *Eco*RI-*Hin*dIII fragments of B2 (Ala-

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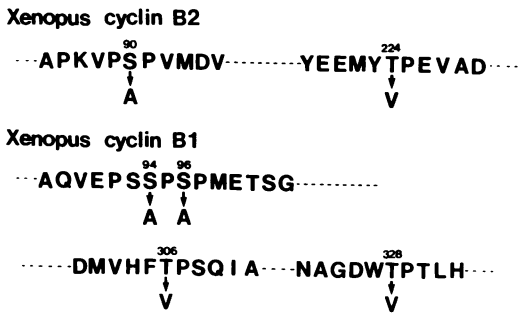

FIG. 1. Sites of in vitro mutagenesis in *Xenopus* cyclins B1 and B2. Numbers above amino acids correspond to the numbering previously reported by Minshull et al. for the amino acid sequences of *Xenopus* cyclins B1 and B2 (23).

90) (0.65 kb) and the mutated *Hin*dIII-*Eco*RI fragments of B2 (Val-224) (0.75 kb) were then inserted into the *Eco*RI site of pGEM1 to make cyclin B2 cDNA mutated at both B2 Ala-90 and Val-124. Similarly, the *Eco*RI-*Sac*I fragments of B1 (Ala-94, Ala-96) (0.4 kb) and the *Sac*I-*Bam*HI fragments of B1 (Val-306, Val-328) (1.0 kb) were inserted into the *Eco*RI and *Bam*HI sites of pGEM1 to make cyclin B1 cDNA mutated at all four sites. The resultant mutant B1 and B2 plasmids were designated pGEMB1* and pGEMB2*. These mutations eliminate all of the Ser and Thr residues followed by Pro, a motif which forms an essential part of the consensus sequence recognized by *cdc2* kinase (25, 37).

Expression of *Xenopus* cyclins in *Escherichia coli*. The normal B1 and B2 expression plasmids, kindly provided by Tim Hunt and Jeremy Minshull, were designated $pET_{3b}B2$ and pET_{3d}-B1 EGEEF, respectively (24). To construct the mutated cyclin B2 expression plasmid, the *Bst*XI-*Sac*I fragments of cyclin B2 cDNA in $pET_{3b}B2$ (1.2 kb) were replaced by the counterpart fragment of pGEM B2* (pET_{3b} B2*). To construct the mutated cyclin B1 expression plasmid, the *Sty*I-*Bgl*II fragments of pGEM B1* (0.9 kb), the *Sal*I-*Bam*HI fragments of pET_{3d}-B1 EGEEF (4.2 kb), and the *Sal*I-*Sty*I fragments of pET_{3d}-B1 EGEEF (0.5 kb) were ligated together. The resulting construct (pET_{3d}-B1*) has the correct N terminus of *Xenopus* cyclin B1 but lacks 56 residues from its normal C terminus and ends with the noncyclin sequence PAANKARKEAELAAATAEQ, starting at amino acid 342. The normal counterpart of cyclin B1 (pET_{3d}-B1) was constructed in the same way. The cyclin expression plasmids were transformed into *E. coli* BL21 (DE3) (39), and cyclin proteins were expressed and purified as described previously (10, 34).

Anticyclin antibodies. Antibodies against cyclins B1 and B2 expressed in bacteria were produced in sheep by using cyclins purified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Anti-cyclin B1 antibodies were purified by affinity chromatography on a column of bacterially synthesized cyclin coupled to activated CH-Sepharose 4B as described by Harlow and Lane (12). Western immunoblotting with anticyclin antibodies was performed as described previously (10).

In vitro transcription and translation. Synthetic mRNAs were transcribed from cDNA subcloned into pGEM1 vectors. Capped transcripts were synthesized by using T7 RNA polymerase as described previously (30). RNAs were translated in an mRNA-dependent reticulocyte lysate system (Promega) according to the manufacturer's instructions.

mRNA-dependent CSF-arrested extracts. The extracts were prepared as described by Murray et al. (27, 28), with the following modifications. The cytostatic factor (CSF)-arrested (metaphase) extracts (28, 38) were incubated with boiled RNase A (final concentration, 1.0 μg/ml; Boehringer Mannheim) at 10°C for 20 min and then incubated with 6,000 U of placental RNase inhibitor (RNasin; U.S. Biochemical) per ml and 1.4 mM dithiothreitol at 10°C for 10 min. These modifications were found necessary for destruction of all of the endogenous mRNA in egg extracts as judged by incorporation of [^{35}S]methionine (see Fig. 5). After addition of calf liver tRNA to 50 μg/ml, 0.02 volume of a concentrated amino acid mixture (Promega) containing [^{35}S]methionine (ICN) to 0.4 mCi/ml, and 0.19 volume of nuclease-treated rabbit reticulocyte lysate, the extract was incubated at 23°C in the presence of 0.15 mM $CaCl_2$. After 40 min, cyclin mRNA was added to 20 to 40 μg/ml. Although the extract showed cell cycle oscillations in the absence of reticulocyte lysate, we found that the addition of reticulocyte lysate significantly increased the translational activity of the extracts and improved the oscillatory behavior. To examine the morphology of sperm nuclei, nuclei were added to the extracts at 10 min after calcium treatment at a final concentration of 7×10^4 per ml and examined as described previously (19).

Phosphorylation and immunoprecipitation of synthesized cyclin proteins in mRNA-dependent CSF-arrested extracts. Cyclin mRNA and 5 mCi of $^{32}P_i$ (ICN) were added to 250 μl of mRNA-dependent CSF-arrested extracts at 40 min after $CaCl_2$ addition. The $^{32}P_i$ added is rapidly converted to [γ-^{32}P] ATP by the extracts. Since the exact time course of the cell cycle varied with different extracts, one-third of each extract was removed and frozen in an ethanol-dry ice bath at 80, 100, and 120 min after $CaCl_2$ addition. The aliquots were then stored at −70°C. After checking of the time course of the cell cycle in the extracts by histone H1 kinase assays and Western blotting with anticyclin antibodies, the aliquots that showed neither high mitotic H1 kinase activity nor cyclin degradation were combined and used for the immunoprecipitation experiments. For immunoprecipitation, the extracts were diluted sixfold in lysis buffer containing 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 1% Triton X-100, 0.1% SDS, 200 mM NaCl, 100 mM NaF, 10 mM $NaPP_i$, 2 mM Na_3VO_4, 50 mM β-glycerophosphate, 5 mM EDTA, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM dithiothreitol, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride and incubated with 300 μl of 10% (vol/vol) Omnisorb (Calbiochem) at 4°C for 1 h in order to remove nonspecific binding materials. After centrifugation at 12,000 × *g*, the supernatant was incubated with 1/200 volume of anti-cyclin B2 serum or 10 μg of affinity-purified anti-cyclin B1 per ml at 4°C for 2 h with or without blocking of the serum by 40 μg of bacterially synthesized cyclin protein. The antibodies were immobilized on Omnisorb at 4°C for 1 h, and the immunoprecipitates were washed twice with the lysis buffer described above, twice with the lysis buffer containing 1 M NaCl, and once with the lysis buffer containing 0.1% Triton X-100. Immunoprecipitates were then eluted with SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis as described by Anderson et al. (1). Phosphoamino acid analysis was performed as described previously (3, 13).

Protein kinase assays with bacterially expressed cyclin proteins. Highly purified MPF was taken through Mono S chromatography as described by Lohka et al. (18), and MPF kinase assays were performed as described previously (9).

Xenopus MAP kinase was purified as described elsewhere (2). MAP kinase assays were performed in a final volume of 30 μl containing 20 mM HEPES, pH 7.0, 5 mM β-mercaptoethanol, 0.1 mg of bovine serum albumin per ml, 5 mM $MgCl_2$, 100 μM ATP, and 30 μCi of [γ-^{32}P]ATP (ICN).

Microinjection of mRNA into *Xenopus* oocytes. Stage VI oocytes from *Xenopus laevis* frogs primed 3 days previously with 35 IU of pregnant mare serum gonadotrophin were manually dissected from their follicular envelopes on the ovary and cultured in modified OR-2 medium containing 10 mM $NaHCO_3$, pH 7.8. Fifty nanoliters of mRNA solution was microinjected into each oocyte. Germinal vesicle breakdown (GVBD) was judged by the appearance of a well-defined white spot at the animal pole, as confirmed by dissection after trichloroacetic acid treatment in doubtful cases. For histone H1 kinase assays, injected oocytes were lysed in buffer (8.4 μl per oocyte) containing 20 mM HEPES, pH 7.5, 80 mM β-glycerophosphate, 15 mM $MgCl_2$, 20 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg of leupeptin per ml and centrifuged for 3 min at 15,000 × *g*. Three microliters of clear supernatant was used per assay under the conditions described (9).

RESULTS

Phosphorylation of cyclins B1 and B2 in mRNA-dependent CSF-arrested extracts. A number of proteins have been reported to be substrates for *cdc2* kinase in vitro. Examination of the phosphorylation sites has shown that the acceptor for serine or threonine is N terminal to a proline, and usually a basic residue is C-terminal or N-terminal to the site (25, 37). Since cyclin is autophosphorylated by *cdc2* kinase in purified MPF (18) as well as when supplied as an exogenous substrate (10), Ser-Pro or Thr-Pro sites are likely candidates for the phosphorylation sites in cyclin. Therefore, we mutated all of the Ser and Thr residues followed by Pro in *Xenopus* cyclins B1 and B2. The resultant mutant cyclin B2 (designated cyclin B2*) contained Ala-90 and Val-224 in place of Ser-90 and Thr-224. Similarly, in the mutant cyclin B1 (cyclin B1*), Ser-94 and Ser-96 were replaced with Ala, and Thr-306 and Thr-328 were replaced with Val (Fig. 1).

First, we examined whether these mutated sites were the real phosphorylation sites in metaphase-arrested (CSF) extracts of unfertilized eggs that reenter the cell cycle in a calcium- and mRNA-dependent manner. Although not quite equivalent to phosphorylation in vivo, egg extracts can be induced to oscillate between M-phase and DNA synthesis in vitro, as judged by nuclear morphology, MPF activity, H1 kinase activity, and cyclin synthesis and degradation (19, 23, 24, 27, 28). Therefore, phosphorylation changes in the extracts almost certainly reflect in vivo situations, and furthermore, since the endogenous cyclin proteins are degraded when the metaphase extracts are induced to enter interphase by calcium addition, this system is appropriate for determining the functional activity of mutant cyclins in the absence of any other cyclin molecules. After RNase treatment to block endogenous cyclin synthesis and calcium addition to stimulate cyclin degradation and exit from M-phase, the extracts were labeled with $^{32}P_i$, and new cyclin proteins synthesized by added mRNA were immunoprecipitated with anticyclin antibodies. When wild-type cyclin B2 mRNA was added, two major phosphoproteins of 54 and 34 kDa were specifically immunoprecipitated (Fig. 2A, lanes a and b). The 54-kDa protein is considered to be cyclin B2, since it has been reported to migrate on Anderson gels (1) with similar

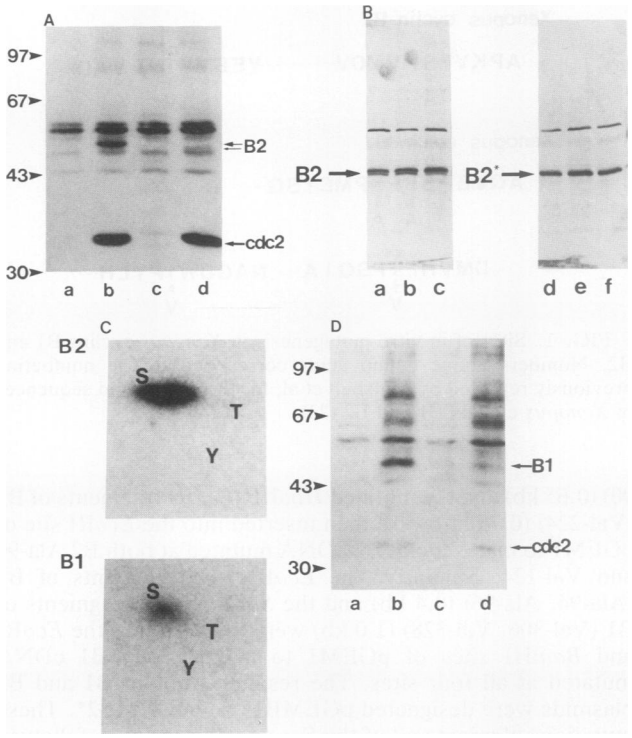

FIG. 2. Phosphorylation of wild-type and mutant cyclins B1 and B2 in mRNA-dependent CSF-arrested extracts. (A) mRNA-dependent CSF-arrested extracts were induced to reenter the cell cycle by the addition of $CaCl_2$ as described in Materials and Methods. At 40 min after calcium treatment, $^{32}P_i$ and either wild-type (lanes a and b) or mutant (lanes c and d) cyclin B2 mRNA was added. Labeling was stopped at 80, 100, and 120 min, and all three fractions were combined and immunoprecipitated with anti-cyclin B2 antibodies with (lanes a and c) or without (lanes b and d) blocking by bacterially expressed cyclin B2 protein. The positions of cyclin B2 protein and *cdc2* kinase are indicated with arrows. (B) Aliquots were removed from the samples described above, in which the labeling was stopped at 80 (lanes a and d), 100 (lanes b and e), and 120 (lanes c and f) min. The expression levels of wild-type (lanes a to c) and mutant (lanes d to f) cyclin B2 protein were determined by Western blotting with anti-cyclin B2 antibodies. (C) CSF-arrested extracts without RNase treatment were labeled with $^{32}P_i$ as for panel A and immunoprecipitated with anticyclin antibodies. Phosphoamino acid analyses of endogenous cyclin B2 (top) and B1 (bottom) were performed. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. (D) Newly synthesized cyclin B1 in an mRNA-dependent CSF-arrested extract was labeled with $^{32}P_i$ as described for panel A. Wild-type B1 is shown in lanes a and b; mutant B1* is shown in lanes c and d. Cyclin B1 proteins were immunoprecipitated with anti-cyclin B1 antibodies with (lanes a and c) and without (lanes b and d) blocking by bacterially expressed cyclin B1 proteins. Sizes are shown in kilodaltons.

mobility (10, 23, 24), and it was not present in blocked immunoprecipitates. Just below this band, a faint 51-kDa phosphoprotein could also be detected, which is compatible with previous reports showing that cyclin B2 is detected as a doublet (8, 10, 23, 24). The 34-kDa protein is *cdc2* kinase, which is well known to become complexed with cyclin, after which it undergoes phosphorylation on tyrosine and threonine residues (38). On the other hand, in mutant cyclin B2* mRNA-dependent extracts, only the faint 51-kDa phosphoprotein could be detected, whereas the level of the 34-kDa phosphoprotein was equivalent to that of wild-type

immunoprecipitates (Fig. 2A, lanes c and d). In both extracts, analysis of *cdc2* kinase eluted from the band showed that it was phosphorylated equally on both tyrosine and threonine residues (data not shown). Recently, Solomon et al. (38) have shown that phosphorylation of *cdc2* kinase can occur only after binding to cyclin. Therefore, the fact that almost equal amounts of phosphorylated *cdc2* kinase were coimmunoprecipitated by anti-cyclin B2 antibodies from each extract strongly suggests that equivalent amounts of cyclin B2 protein were synthesized and that phosphorylation of cyclin is not required for *cdc2* kinase phosphorylation. As a further test, we confirmed by Western blotting that both wild-type and mutant cyclin B2 were synthesized to the same extent in these extracts (Fig. 2B). Again, wild-type cyclin B2 was detected as a doublet, and the proportion of the upper band increased with time, whereas mutant cyclin B2 was detected as a single band (Fig. 2B). These results clearly indicate that the dramatically reduced phosphorylation of mutant cyclin B2 was not due to a lower amount of the newly synthesized mutant B2 in the extract. Phosphoamino acid analysis revealed that both endogenous cyclin B2 (Fig. 2C, top) and newly synthesized wild-type cyclin B2 (data not shown) were phosphorylated on serine residues. In addition, both the lower band of wild-type cyclin B2 and mutated cyclin B2 were also weakly phosphorylated on serine residues (data not shown).

Similar results were obtained with wild-type and mutant cyclin B1. *cdc2* kinase (34 kDa) was also coimmunoprecipitated with cyclin B1 (50 kDa). Phosphorylation of mutant cyclin B1 was dramatically reduced (Fig. 2D), although the two cyclins were synthesized to the same extent (data not shown). However, phosphorylation of wild-type cyclin B1 did not retard electrophoretic mobility on gels. The phosphorylation occurred on serine residues in all cases, endogenous (Fig. 2C, bottom), newly synthesized wild-type, and mutant cyclin B1 (data not shown).

From these results, we concluded that (i) the main phosphorylation sites of *Xenopus* cyclins in the extracts are Ser-90 in B2 and Ser-94 or Ser-96 in B1, whereas other threonine residues and serine residues are not significantly phosphorylated, and (ii) phosphorylation of Ser-90 in cyclin B2 causes electrophoretic retardation, whereas phosphorylation of Ser-94 or Ser-96 in B1 does not.

Phosphorylation of bacterially expressed cyclins B1 and B2 by purified MPF and MAP kinase in vitro. One kinase likely to be responsible for B-type cyclin phosphorylation in extracts is *cdc2* kinase, which is known to combine with B-type cyclins to form active MPF (10, 18, 22, 31). Previously, we have shown that purified MPF can phosphorylate bacterially expressed *Xenopus* cyclins B1 and B2 in vitro (10) as well as cyclin associated with *cdc2* kinase in the MPF complex (18). Therefore, we examined whether bacterially expressed mutant cyclin proteins could be substrates for purified MPF. Wild-type cyclin B2 protein phosphorylated by MPF exhibited a retarded electrophoretic mobility relative to the position of the Coomassie-stained band and had the same mobility as phosphorylated endogenous cyclin B2 in the MPF preparation, which could be seen after longer exposure (Fig. 3A, lanes c and e). In contrast, the mutant B2 protein was not detectably phosphorylated in vitro by MPF (Fig. 3A, lane d). Similarly, wild-type cyclin B1 protein was phosphorylated by MPF, whereas mutant B1 was not (Fig. 3A, lanes a and b). Interestingly, both wild-type cyclin B1 and B2 proteins were phosphorylated in vitro on threonine as well as serine residues (Fig. 3B), in contrast to the situation in the extracts in which only phosphoserine was detected (Fig. 2C).

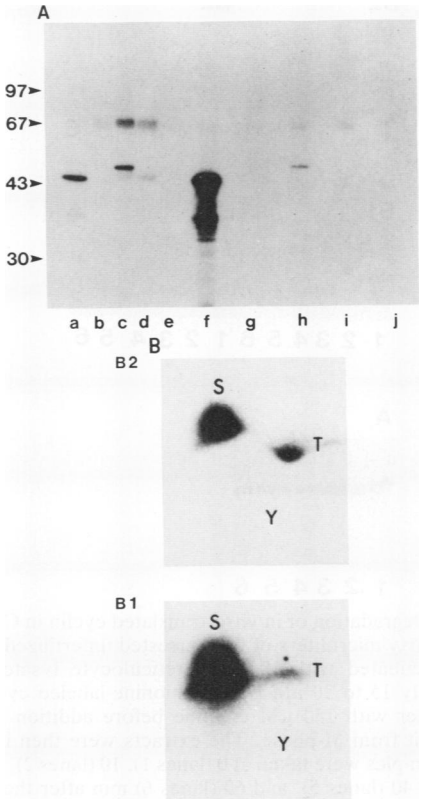

FIG. 3. Phosphorylation of bacterially expressed cyclin proteins by *Xenopus* MPF and MAP kinase in vitro. Purified MPF (lanes a to e) and purified MAP kinase (lanes f to j) were incubated with 2 μg of wild-type B1 (lanes a and f), mutant B1 (lanes b and g), wild-type B2 (lanes c and h), mutant B2 (lanes d and i), or no exogenous cyclin (lanes e and j). The proteins were analyzed on a 10% polyacrylamide gel prepared as described by Laemmli (15). (A) Autoradiographs; (B) phosphoamino acid analysis of wild-type B1 and B2 phosphorylated by MPF. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

Next, we determined whether another egg kinase such as MAP kinase could phosphorylate cyclin protein. This kinase is known to be activated during oocyte maturation and during the first embryonic cell cycle in *X. laevis* (2, 7). We found that cyclin B1 was very heavily phosphorylated by purified MAP kinase, whereas B2 was not significantly phosphorylated (Fig. 3A, lanes f and h). Again, mutant cyclins B1 and B2 were not phosphorylated (Fig. 3A, lanes g and i). Therefore, MAP kinase also appears to recognize the Ser/Thr-Pro motif in some substrates, as also reported recently for the MAP kinase phosphorylation site in myelin basic protein (5). The latter site is remarkably similar in primary structure to Ser-94 in cyclin B1.

Cyclin degradation in CSF-arrested extracts. One possible role of cyclin B phosphorylation is to affect its ability to undergo subsequent destruction at the metaphase/anaphase transition. To test this hypothesis, we incubated cyclin proteins translated in an mRNA-dependent rabbit reticulocyte lysate with CSF-arrested extracts in which resynthesis of cyclin was prevented by emetine treatment. After calcium addition, both wild-type cyclins B1 and B2 were rapidly

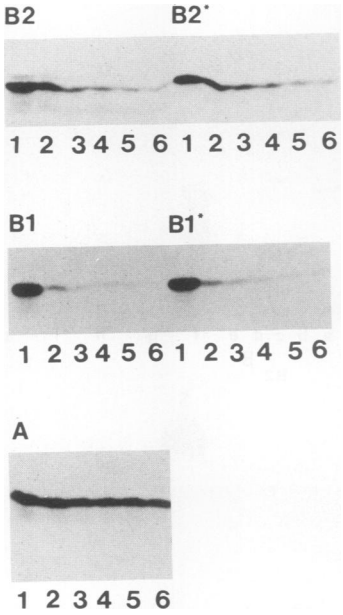

FIG. 4. Degradation of in vitro-translated cyclin in CSF-arrested extracts. Thirty microliters of CSF-arrested unfertilized egg extract (28) was incubated with 10 μl of reticulocyte lysate containing approximately 15 to 20 nM [^{35}S]methionine-labeled cyclin as indicated together with 150 μM emetine before addition of $CaCl_2$ to stimulate exit from M-phase. The extracts were then incubated at 23°C, and samples were taken at 0 (lanes 1), 10 (lanes 2), 20 (lanes 3), 30 (lanes 4), 40 (lanes 5), and 60 (lanes 6) min after the addition of calcium and analyzed by SDS-gel electrophoresis for the level of cyclin remaining.

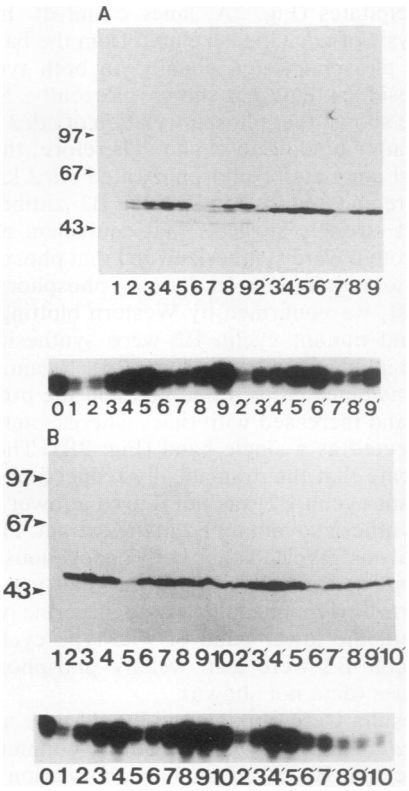

FIG. 5. Cyclin synthesis and histone H1 kinase activity in mRNA-dependent CSF-arrested extracts. (A) mRNA-dependent CSF-arrested extracts were incubated with [^{35}S]methionine and induced to reenter the cell cycle by the addition of $CaCl_2$. At 40 min after calcium treatment, the extracts were split and the halves were incubated with mRNA of either wild-type (lanes 2 to 9) or mutant (lanes 2′ to 9′) cyclin B2. Samples were taken every 20 min and analyzed for ^{35}S-labeled protein synthesis (top) and histone H1 kinase activity (bottom). (B) The experiments were performed as for panel A with mRNA of wild-type (lanes 2 to 10) or mutant (lanes 2′ to 10′) cyclin B1. In both panels, samples were taken at 0 min before $CaCl_2$ addition (lanes 0, for histone H1 kinase assay only) and then at 40 (lanes 1), 60 (lanes 2 and 2′), 80 (lanes 3 and 3′), 100 (lanes 4 and 4′), 120 (lanes 5 and 5′), 140 (lanes 6 and 6′), 160 (lanes 7 and 7′), 180 (lanes 8 and 8′), 200 (lanes 9 and 9′), and 220 (lanes 10 and 10′ [only in panel B]) min after the addition of $CaCl_2$.

degraded as reported previously (28) (Fig. 4). Similarly, both mutant cyclins B1 and B2 were degraded with the same kinetics as those of the wild-type cyclins (Fig. 4). Therefore, phosphorylation of cyclin B1 or B2 at these sites does not appear to be important in their degradation, at least in exit from meiosis II. Interestingly, *Xenopus* cyclin A, another type of cyclin, was not degraded as efficiently as either B-type cyclin. At 40 min after release from M-phase arrest, 50% of the cyclin A protein remained; for cyclins B1 and B2, only 4 and 22%, respectively, was left (Fig. 4).

Cyclin induction of mitosis in mRNA-dependent CSF-arrested extracts. Another possible role for cyclin phosphorylation is in regulation of *cdc2* kinase activity. Therefore, we examined mRNA-dependent CSF-arrested extracts for sperm nuclear morphology, cyclin synthesis, and histone H1 kinase activity, the latter thought to reflect *cdc2* kinase activity. We confirmed that no protein was synthesized during the time after release from metaphase by calcium treatment until mRNA addition (Fig. 5A and B, top, lanes 1) and that proteins other than cyclin were not synthesized after mRNA addition (Fig. 5A and B, top, lanes 2 to 10 and 2′ to 10′). Either wild-type or mutant cyclin B2 mRNA induced two cycles of activation of *cdc2* histone H1 kinase activity in a manner dependent on cyclin synthesis (Fig. 5A). In addition, the nuclear envelopes of pronuclei broke down and chromosomes condensed in both extracts (data not shown), indicating that a true mitotic state had been produced by both wild-type and mutant cyclin. It is noteworthy that wild-type cyclin B2 was detected as a doublet and that the proportion of the upper band increased throughout interphase, whereas mutant cyclin B2 was a single band throughout the entire cycle (Fig. 5A, top). After mitosis, both wild-type and mutant cyclin B2 protein levels and the *cdc2* histone H1 kinase level declined dramatically. Similarly, mutant cyclin B1 could activate histone H1 kinase activity (Fig. 5B), induce nuclear envelope breakdown and chromosome condensation (data not shown), and be degraded efficiently (Fig. 5B, top). In this experiment, mutant cyclin B1 mRNA induced only one cell cycle. However, it should be noted that mutant cyclin B1 was not synthesized as efficiently after first mitosis in this extract as was the wild-type protein. Since a threshold amount of cyclin is required for *cdc2* kinase activation, the absence of a second cycle is not indicative of an intrinsic deficiency of mutant B1 for stimulation of M-phase. Furthermore, in some experiments, wild-type mRNA drove fewer cycles than mutant mRNA, and in numerous experiments there was no pattern of difference in induction of mitosis between wild-type and mutant B1 that could not be accounted for by differences in the rate of cyclin translation (data not shown). One possible

TABLE 1. Effect of cyclin mRNA on oocyte maturation

Expt	mRNA injected[a]	No. of oocytes	% GVBD
1	B2	10	90
	B2*	10	90
	B1	14	79
	B1*	8	100
	None	9	0
	10 μM progesterone	9	100
2[b]	B2	20	90
	B2*	23	96
	B1	14	100
	B1*	12	83
	None	14	0
	10 μM progesterone	26	96

[a] The amount of mRNA injected was 125 ng per oocyte. GVBD was assessed 3 to 4 h after injection, and assessment began earlier with cyclin than with progesterone.

[b] In this experiment, histone H1 kinase activity of the oocyte extract was also measured (Fig. 6).

reason for activation of a single cycle by either wild-type or mutant mRNA is the degradation of mRNA due to reactivation of micrococcal nuclease in the reticulocyte lysate component upon addition of calcium to release CSF arrest. The subsequent reduction in the rate of cyclin synthesis could lead to slower rates of *cdc2* kinase activation or levels of cyclin below the threshold for activation of *cdc2* kinase.

Cyclin induction of oocyte maturation. It is well known that cyclin mRNA from heterologous species can induce oocyte maturation when microinjected in *Xenopus* oocytes (30, 40, 41). In addition, we have recently shown that recombinant cyclin proteins are sufficient to induce maturation in the absence of new protein synthesis (35). It is possible that in the special cell cycles of meiosis I and II, cyclin B phosphorylation plays an important role, because in many respects the cell cycle is different in oocytes and eggs. For example, the *Xenopus* c-*mos* proto-oncogene kinase that is active only in the meiotic cycles of oocyte maturation has been shown to phosphorylate cyclin B2 in vitro (34, 36). Therefore, we tested whether mRNA for mutant cyclins B1 and B2 could induce oocyte maturation. As shown in Table 1, we found that both mutant and wild-type cyclin B1 and B2 mRNAs could induce GVBD. Furthermore, we confirmed that both wild-type and mutant cyclin mRNAs could activate histone H1 kinase activity in injected oocytes (Fig. 6) to levels even greater than that observed at GVBD in controls treated with progesterone.

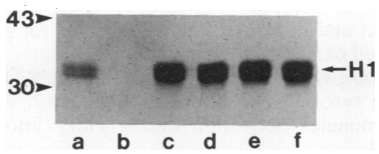

FIG. 6. Effect of cyclin mRNA injection on histone H1 kinase activity in oocytes. In experiment 2 of Table 1, three oocytes from each treatment were removed and lysed as described in Materials and Methods. An aliquot of the extracts corresponding to 0.3 oocyte was assayed for kinase activity. Lanes: a, 10 μM progesterone; b, no treatment; c, wild-type B2; d, mutant B2; e, wild-type B1; f, mutant B1.

DISCUSSION

In this report we have shown that Ser-90 of cyclin B2 and Ser-94 or Ser-96 of cyclin B1 are the main phosphorylation sites for these cyclins in *Xenopus* egg extracts. Since this extract reproduces the morphological and biochemical characteristics of the cell cycle (19, 27, 28), the data strongly suggest that these sites are the real phosphorylation sites in vivo. In addition, we have shown that purified MPF phosphorylates exclusively these sites in vitro. Therefore, we conclude that these are the phosphorylation sites of *cdc2* kinase in the cyclin B/*cdc2* MPF complex. For both cyclin B1 and B2 there was a very low level of residual phosphorylation present even in mutant forms (Fig. 2A and D). Whether this represents an additional site(s) of phosphorylation by other kinases or has any functional significance cannot be determined from these data. Although Thr-224 of cyclin B2 and Thr-306 or Thr-328 of cyclin B1 were phosphorylated by purified MPF in vitro, no phosphothreonine was detected in either cyclin in extracts, making it unlikely that these sites are phosphorylated in vivo. Although all of the phosphorylated serine residues are followed by proline, as shown in Fig. 1, the neighboring sequences do not contain polar or basic residues as previously observed in most consensus sequences recognized by *cdc2* kinase (21, 25, 37). The lack of requirement for polar or basic residues for cyclin B phosphorylation could reflect a less stringent consensus for cyclin, since it is normally physically bound to *cdc2* when it is autophosphorylated in the cell. Data presented here suggest similarity in the substrate specificity of MAP kinase and *cdc2* kinase. Both kinases are regulated by dual phosphorylation on tyrosine and threonine residues although with opposite effects on activity. Both phosphorylated cyclin B1 in vitro on both Ser and Thr residues, but phosphorylation in extracts or in vitro was abolished by the mutations. Ser-94 in B1 fits most closely the established site of phosphorylation of myelin basic protein for MAP kinase, but further work is needed to directly identify the site in cyclin B1 phosphorylated by each kinase.

Another important finding of this study is the lack of functional effect on cyclins of mutations that prevent phosphorylation. Mutant cyclins could be degraded as efficiently as wild-type proteins at the end of both meiosis and mitosis. Félix et al. (6) have reported that cyclin proteolysis can be triggered by adding *cdc2* kinase to interphase *Xenopus* extracts and suggested models in which phosphorylation of a cyclin protease or of cyclin itself by *cdc2* kinase regulates degradation of cyclin. However, our data clearly exclude the possibility that phosphorylation of cyclin itself by *cdc2* kinase or MAP kinase is a trigger for its destruction. This conclusion is also consistent with previous findings (8, 20, 24) and present data (Fig. 1B and 5A) showing that the phosphorylated form of cyclin B2 with a retarded electrophoretic mobility can be detected early in interphase. Recently, Lee et al. (16) reported that a form of phosphatase 2A, designated INH, could prevent the spontaneous activation of pre-MPF in ammonium sulfate fractions of oocytes. Moreover, they showed that sea urchin cyclin B labeled with ^{32}P in an egg extract could be dephosphorylated in vitro by INH when H1 kinase activity by the cyclin/*cdc2* complex was also declining. Although a physiological role for INH in cell cycle regulation has not yet been established, the results presented here suggest that effects of INH/phosphatase 2A are not due to dephosphorylation of sites in cyclin fitting the *cdc2* kinase consensus sequence.

Mutant cyclins could also induce mitosis in egg extracts

and resumption of meiosis in oocytes as well as the wild-type protein could. Therefore, cyclin phosphorylation by *cdc2* kinase most likely does not contribute to MPF activation but rather is a consequence of activation of MPF. It is of some interest that the level of *cdc2* H1 kinase activity in oocytes injected with wild-type or mutant mRNAs was significantly higher than with progesterone treatment of control oocytes. We and others have previously shown (35, 38) that introduction of new cyclin proteins into egg extracts or oocytes activates *cdc2* kinase to levels greater than normal metaphase, and this is most likely due to activation of endogenous pre-MPF cyclin/*cdc2* complexes as well as recruitment of additional monomeric *cdc2* molecules into activated complexes with the introduced cyclin species.

We have shown that a single species of B-type cyclin could induce mitosis and resumption of meiosis, which is consistent with previous findings (23) demonstrating the necessity of ablating the mRNA for both cyclins B1 and B2 to prevent entry into mitosis in *Xenopus* egg extracts. Currently, we do not understand the differential roles of cyclins B1 and B2 or cyclin A. Cyclin A has been reported to behave differently from B-type cyclin to some extent (17, 24, 33). In particular, cyclin A has been suggested to bind other kinases distinct from *cdc2* in human cultured cells (33), but in *Xenopus* egg extracts, available evidence indicates all three cyclins bind *cdc2* kinase itself and exhibit similar substrate specificity (24). Although each cyclin complex exhibits different kinetics of activation in egg extracts, the results in this report suggest that these differences are not due to altered phosphorylation of the cyclin B component by MAP kinase or *cdc2* kinase itself. A surprising result from the work described here was the discovery that a substantial fraction of cyclin A (50%) was not degraded upon exit from meiosis II in CSF extracts. This finding suggests that this level of cyclin A is insufficient to cause M-phase, although we have not yet demonstrated that the residual cyclin A is associated with *cdc2* kinase. Further work examining the potential role of phosphorylation in the function of cyclin A and regulatory differences between A- and B-type cyclins is clearly warranted.

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