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Requirement of *mos*^{Xe} Protein Kinase for Meiotic Maturation of *Xenopus* Oocytes Induced by a *cdc2* Mutant Lacking Regulatory Phosphorylation Sites

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The p34^{cdc2} protein kinase is a component of maturation-promoting factor, the master regulator of the cell cycle in all eukaryotes. The activity of p34^{cdc2} is itself tightly regulated by phosphorylation and dephosphorylation. Predicted regulatory phosphorylation sites of *Xenopus* p34^{cdc2} were mutated in vitro, and in vitro-transcribed RNAs were injected into *Xenopus* oocytes. The *cdc2* single mutants Thr-14→Ala and Tyr-15→Phe did not induce germinal vesicle breakdown (GVBD) upon microinjection into oocytes. In contrast, the *cdc2* double mutant Ala-14/Phe-15 did induce GVBD. Both the Ala-14 and Ala-14/Phe-15 p34^{cdc2} mutants were shown to coimmunoprecipitate cyclin B1 and to phosphorylate histone H1 in immune complex kinase assays. Microinjection of antisense oligonucleotides to c-*mos*^{Xe} was used to demonstrate the role of *mos* protein synthesis in the induction of GVBD by the Ala-14/Phe-15 *cdc2* mutant. Thr-161 was also mutated. p34^{cdc2} single mutants Ala-161 and Glu-161 and triple mutants Ala-14/Phe-15/Ala-161 and Ala-14/Phe-15/Glu-161 failed to induce GVBD in oocytes and showed a decreased binding to cyclin B1 in coimmunoprecipitations. Each of the *cdc2* mutants was also assayed by coinjection with cyclin B1 or c-*mos*^{Xe} RNA into oocytes. Several of the *cdc2* mutants were found to affect the kinetics of cyclin B1 and/or *mos*-induced GVBD upon coinjection, although none affected the rate of progesterone-induced maturation. We demonstrate here the significance of Thr-14, Tyr-15, and Thr-161 of p34^{cdc2} in *Xenopus* oocyte maturation. In addition, these results suggest a regulatory role for *mos*^{Xe} in induction of oocyte maturation by the *cdc2* mutant Ala-14/Phe-15.

The p34^{cdc2} protein kinase is a critical component in cell cycle regulation in eukaryotic cells. In *Xenopus laevis*, p34^{cdc2} is one subunit of maturation-promoting factor (MPF), a cytoplasmic factor that is active in mitosis and inactive in interphase (9, 20). The other subunit of MPF is cyclin B (19). The synthesis and degradation of cyclin regulates the cell cycle in early embryos (47), but its association with p34^{cdc2} is insufficient for activation of MPF (51, 52). MPF activation has also been shown to be regulated by phosphorylation and dephosphorylation of p34^{cdc2}. Phosphorylation of amino acids Thr-14 and Tyr-15 occurs only after binding of p34^{cdc2} to cyclin B (43, 50, 60, 61) and is a negative regulatory event that keeps the p34^{cdc2}/cyclin complex in an inactive state (23, 34, 48, 61). The p107^{wee1} and *mik1* protein kinases are likely to be involved in the inactivating phosphorylation of both Thr-14 and Tyr-15 in yeast cells (10, 41, 55). An activating dephosphorylation of both Thr-14 and Tyr-15 appears to be caused by the protein phosphatase *cdc25*, as indicated by in vitro and in vivo results (8, 21, 37, 55). *CDC28* is the *Saccharomyces cerevisiae* homolog of the *Schizosaccharomyces pombe cdc2* gene (1, 62). The *CDC28* mutants Ala-18/Phe-19 and Phe-19 do not initiate premature mitosis in *S. cerevisiae*, suggesting a novel method of *cdc2* regulation in this organism (1, 62). Phosphorylation on a second Thr of p34^{cdc2}, probably Thr-161 of the *Xenopus* homolog of *S. pombe* p34^{cdc2}, is necessary for an active protein complex (5, 24, 60, 61). This conclusion is based on results from mutagenic analysis as well as in vitro results demonstrating that Thr dephosphorylation can inactivate MPF (5, 24, 60, 61). Although the kinase responsible for phosphorylation of Thr-161 has not yet been identified, an activity which can catalyze the dephosphorylation of Thr-161 in p34^{cdc2}, called INH, has been isolated from *Xenopus* oocytes (38, 60). INH, a negative regulator of MPF activity, contains a form of protein phosphatase 2A (38, 60).

Immature *Xenopus* oocytes are naturally arrested at the G_2/M border of meiosis I and contain an inactive p34^{cdc2} bound to cyclin (18, 32). p34^{cdc2} in this pre-MPF complex is phosphorylated on Tyr-15 (9, 12, 30). Pre-MPF may also be phosphorylated on Thr-14 (21, 35, 37). Oocytes naturally stimulated by progesterone progress through meiosis I and are then arrested at metaphase of meiosis II. Following stimulation by progesterone, Tyr-15 and possibly Thr-14 are dephosphorylated (9, 12, 30), and active MPF is formed (22). MPF activity cycles during oocyte maturation, decreasing during the meiosis I/meiosis II transition and then increasing as metaphase II begins. MPF then remains in an active state until fertilization, at which time cyclin is degraded, resulting in inactivation of the complex (47). There is an excess of monomeric p34^{cdc2} present in the immature or resting oocyte (32), and injection of *cdc2* RNA into *Xenopus* oocytes does not induce germinal vesicle breakdown (GVBD) (13). GVBD, characterized by the appearance of a white spot on the dark animal pole of *Xenopus* oocytes, is indicative of entry into meiosis I and formation of active MPF (9, 42). Although cyclin synthesis is not necessary for induction of GVBD by progesterone, injection of cyclin protein can induce GVBD even in the absence of protein synthesis (22, 46, 53). Another protein which can induce GVBD in resting oocytes in the absence of protein synthesis is *cdc25* (21, 37). Results obtained in vitro suggest that injection of *cdc25* is likely to induce GVBD by the dephosphorylation of p34^{cdc2} on Thr-14 and Tyr-15. One model for pre-MPF activation

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suggests that pre-MPF may be a complex of cyclin and p34^{cdc2} that is phosphorylated on Thr-14 and Tyr-15 (35, 37). Pre-MPF may also be phosphorylated on Thr-161 of p34^{cdc2}, or this event may rapidly follow dephosphorylation of both Thr-14 and Tyr-15 (37). The sum of these events would activate pre-MPF.

In addition to *cdc25*, a number of other proteins and RNAs which can induce GVBD have been identified. An interesting example is the *mos* protein, a germ cell-specific serine/threonine protein kinase (14, 64). Microinjection of in vitro-transcribed *mos* RNA results in meiotic maturation (15, 16, 56). Induction of GVBD by *mos* does not require synthesis of other proteins, as injection of a bacterially expressed *mos* protein into resting oocytes in the presence of cycloheximide results in GVBD (65). Interestingly, this protein was unable to induce meiosis II in the presence of cycloheximide, suggesting the requirement for synthesis of some other protein for this event (65). Microinjection of antisense oligonucleotides to c-*mos*^{Xe} RNA demonstrates that *mos*^{Xe} is required for progesterone- or insulin-induced maturation (57). *mos* is also required for the transition from meiosis I to meiosis II (4, 31). A second function for *mos* was found in its identification as a component of cytostatic factor (CSF) (58). CSF was initially characterized as a protein fraction isolated from unfertilized eggs which could arrest cell division when microinjected into a dividing two-cell embryo (42). As CSF activity involves stabilization of active MPF, it is possible that *mos*^{Xe} plays a role in regulating the phosphorylation state of p34^{cdc2} and/or cyclin. Recently, another role for *mos* was found in its ability to phosphorylate and bind to tubulin (66). Although the significance of this phosphorylation is unknown, phosphorylation of tubulin on Ser has been demonstrated to affect the ability of tubulins to polymerize (63). In addition, the localization of *mos* to the microtubule network may be helpful in identifying its potential substrates.

In this study, 11 p34^{cdc2} mutants affecting Thr-14, Tyr-15, and Thr-161 were constructed. The mutant RNAs were microinjected into *Xenopus* oocytes and then assayed for induction of GVBD, histone H1 kinase activity, and cyclin B1 binding. Each of the mutated amino acids was found to be significant in the regulation of p34^{cdc2} activity. These mutants were found to differ in the ability to bind cyclin B1 and to induce GVBD in the presence of coinjected RNAs encoding cyclin B1 and c-*mos*^{Xe}. The p34^{cdc2} Thr-161 mutants showed a decrease in binding to cyclin B1 in vitro but appeared to maintain some in vivo activity. One p34^{cdc2} mutant RNA, Ala-14/Phe-15, was found to induce GVBD upon microinjection into *Xenopus* oocytes. Antisense oligonucleotides to *mos*^{Xe} were used to show that this mutant requires synthesis of the *mos* protein for induction of maturation. These results suggest a regulatory role for *mos* in p34^{cdc2} mutant Ala-14/Phe-15-induced oocyte maturation.

MATERIALS AND METHODS

Site-directed mutagenesis. A *cdc2* clone was isolated from an *X. laevis* oocyte cDNA library and subcloned into pBluescript KS(−) (Stratagene). The complete nucleotide sequence was determined, and the predicted amino acid sequence is shown in Fig. 1. The amino acid sequence differs, about 3%, from that of a second clone (Fig. 1) obtained from the same library. The two different *Xenopus cdc2* clones most likely result from the genome duplication in *X. laevis*, as we have seen no difference in expression of the two genes or in cyclin binding properties of their gene products (data not shown). Clone B was used for subsequent experiments and is referred to throughout as simply *cdc2*. Single-stranded uracil-containing *cdc2* DNA was isolated (36) and mutagenized as described previously (67), using the following oligonucleotides:

Thr-161→Ala, GCTTTTGGAATTCCAGTACGCGTTTACGCACACGAGGTCG

Thr-161→Glu, GCTTTTGGAATTCCAGTACGCGTTTACGAACACGAGGTCG

Thr-14→Ala, GAAGATCGGAGAGGGCGCATATGGGGTCGTATACAAGGGTC

Tyr-15→Phe, GATCGGAGAGGGCACATTTGGGGTCGTATACAAGGGTC

Restriction fragments containing the mutated DNAs were sequenced and then subcloned into the appropriate region of wild-type *cdc2* in pSB5 [a pSP64(polyA) derivative]. All of the double mutants except the Ala-14/Phe-15 mutant were made by exchanging restriction fragments of the previously made single mutants. As we found it difficult to create the Ala-14/Phe-15 double mutant by site-directed mutagenesis, this mutant was made by the following method. First, the following complementary oligonucleotides were annealed to generate a restriction fragment: AGCTTCAGTCATGGAC GAGTACACTAAAATAGAGAAGATCGGTGAGGGCGC ATTTGGGGTCGT and ATACGACCCCAAATGCGCCCT CACCGATCTTCTCTATTTTAGTGTACTCGTCCATGAC TGA. This fragment possesses *Hin*dIII and *Acc*I cohesive ends and encodes the start codon and the first 16 amino acids of the *cdc2* gene. This fragment was ligated into the wild-type *cdc2* gene in pSB5 which had been digested with *Hin*dIII in the upstream polylinker and with *Acc*I, which cuts in the *cdc2* coding region. Isolation of the correct insert was verified by DNA sequencing prior to use.

The peptide sequence used in this study as an epitope tag (referred to as TAG) was derived from an internal region of the platelet-derived growth factor receptor. It was attached to the *cdc2* gene as follows. (i) The wild-type *cdc2* gene was mutated to contain a *Bgl*II site just upstream of the stop codon by using the following oligonucleotide: CCTGCCA ATCAGATCAAGATCTAAAACAGCAAACG. This oligonucleotide also mutated Arg-301 to Lys-301 and Gln-302 to Ile-302 in p34^{cdc2}. (ii) A synthetic restriction fragment which encodes the epitope tag was formed by hybridizing the following complementary oligonucleotides: GATCTTGGAG GTCATCGTGGTACCACACAGTCTACCCTTTATGCTC TAGCTGCA and GCTAGAGCATAAAGGGTAGACTGTG TGGTACCACGATGACCTCCAA. (iii) This synthetic restriction fragment was ligated to the *cdc2* gene created in step i, which had been digested at the new *Bgl*II site and also with *Pst*I, which cuts in the downstream polylinker.

In vitro transcription and translation of *cdc2*^{Xe}, cyclin B1, and *mos*^{Xe}. 5′-capped and polyadenylated RNAs were transcribed in vitro from the *cdc2*^{Xe}, cyclin B1, and *mos*^{Xe} genes cloned into pSB5 as described previously (44). RNAs were analyzed by gel electrophoresis and by in vitro translation in rabbit reticulocyte lysates containing 50 μCi of [^{35}S]Met (1,000 Ci/mmol).

Preparation of antipeptide antibodies to *cdc2*^{Xe} and TAG. Cyclin B1 antipeptide antibody preparation has been described elsewhere (13). Peptides corresponding to the carboxy terminus of *cdc2*^{Xe} (LDKSRRLPDNQIRN) and the TAG sequence (KEVIVVPHSLPFML) were synthesized and coupled to bovine serum albumin (BSA) by using glutaraldehyde as described previously (25). Coupled peptides were used to immunize rabbits in conjunction with Freund's adjuvant (25). The reactivities of the resulting

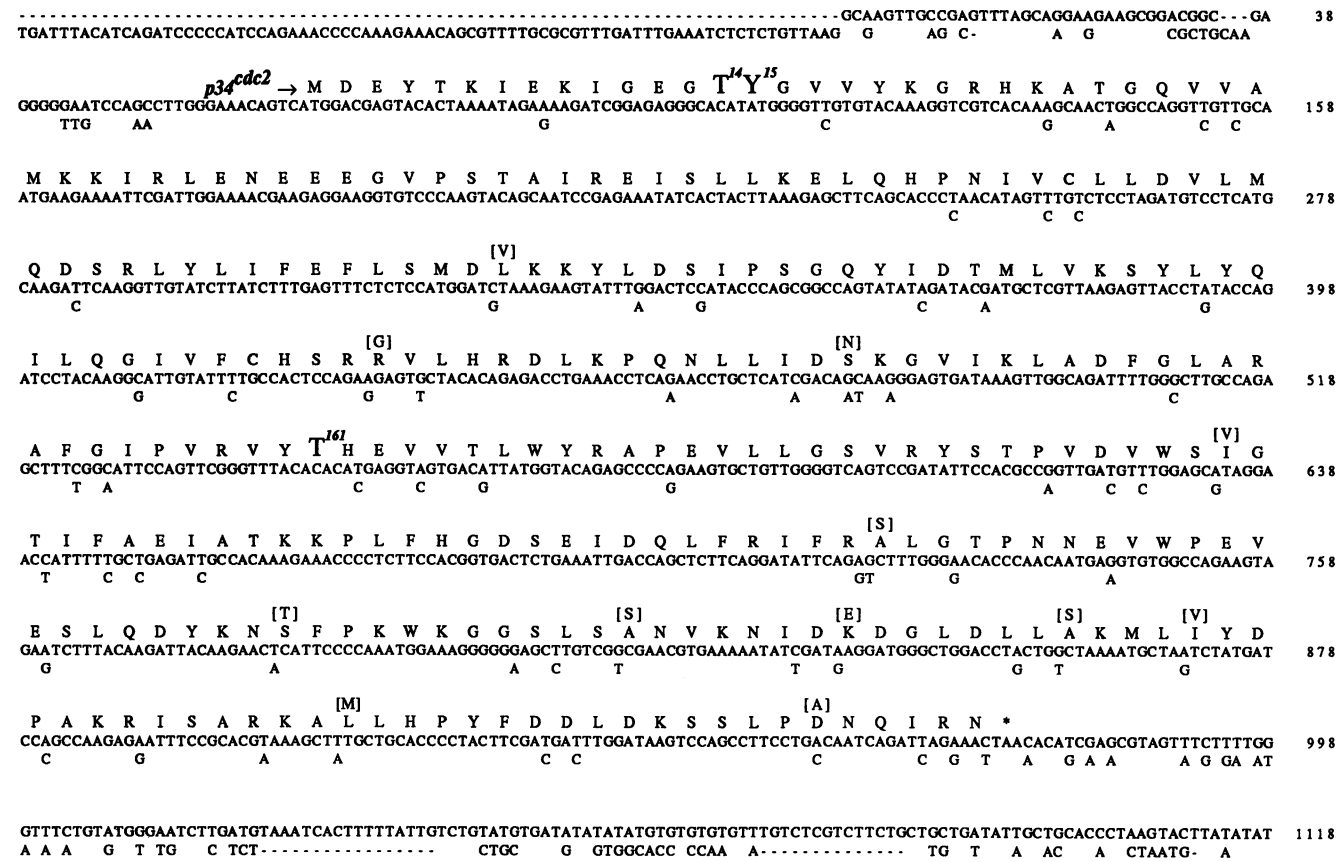

FIG. 1. Nucleotide sequences of *X. laevis cdc2* clones A and B and their predicted protein products. The upper nucleotide sequence corresponds to clone A. The lower nucleotide sequence corresponds to clone B, and only differences with respect to clone A are shown. The predicted amino acid sequence of clone A $p34^{cdc2}$ is shown above the nucleotide sequence of clone A. Amino acids which are different in clone B $p34^{cdc2}$ are shown within square brackets above the sequence of clone A $p34^{cdc2}$. A dash in the nucleotide sequence represents a gap used in alignment. The polyadenylation signal begins at nucleotide 1194. All sequence data for clone A were obtained from a single cDNA clone, designated clone 46. Nucleotides 1 to 1110 of clone B were obtained from clone 9, and the remainder of the clone B sequence was obtained from clone 28. This latter clone was incomplete at the 5′ end but otherwise appeared identical to clone 9, as judged by restriction enzyme mapping and DNA sequencing. Nucleotide numbers shown at the right margin refer to the clone A sequence, which was 1,234 nucleotides in length. The length of the clone B sequence is 1,237 nucleotides. The calculated molecular mass of the clone A protein product is 34.6 kDa, and that of the clone B protein product is 34.4 kDa. Clone B was used for the mutagenesis experiments described in this work and is referred to simply as *cdc2*. The residues examined by mutagenesis in this work, Thr-14, Tyr-15, and Thr-161, are highlighted in the amino acid sequence.

antisera were analyzed by immunoprecipitation of in vitro-transcribed and -translated proteins and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The crude anti-TAG serum was affinity purified on a column of the peptide coupled to Affi-Gel 10 (Bio-Rad, Richmond, Calif.) and reassayed for reactivity prior to use.

Oocyte microinjections and immunoprecipitations. Stage VI oocytes (6) were either treated with progesterone (30 μM) or injected with in vitro-synthesized RNA (50 to 100 ng in a volume of 50 nl). Oocytes were then incubated in modified Barth's saline (MBS-H) (59) containing 0.5 mCi each of [^{35}S]Met and [^{35}S]Cys per ml [MBS-H is 88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4]. For immunoprecipitations, oocytes were lysed at approximately 50% GVBD ($GVBD_{50}$) (3 to 4 h). GVBD was monitored by the appearance of a white spot in the animal hemisphere. Oocytes were checked every 30 min to determine rate of maturation. GVBD was verified by fixation in 5% trichloroacetic acid followed by manual dissection where indicated.

For immunoprecipitations, oocytes were lysed in 20 μl per oocyte in immunoprecipitation buffer [8.5 mM Tris HCl (pH 6.8), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 50 mM β-glycerophosphate, 10 mM NaF, 2 mM ATP, 2 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM ethylene glycerol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.5 μg of leupeptin per ml, 10 μg of aprotinin per ml, 10 μM pepstatin A] and frozen. Oocytes were later thawed, lysed, and centrifuged, and the clear supernatant was collected for immunoprecipitation. Lysates were precleared with protein A-Sepharose and then immunoprecipitated with either anti-cyclin B1 serum or anti-TAG serum as indicated in the figure legends. The immune complexes were

analyzed by SDS–15% PAGE and fluorography followed by autoradiography.

For antisense experiments, 50 nl of a 2-mg/ml solution of antisense *mos*^{Xe} oligonucleotide (16) was microinjected, and oocytes were incubated for 4 h prior to injection of *cdc2* Ala-14/Phe-15 mutant RNA or incubation in 30 μM progesterone.

Histone H1 kinase assays. Stage VI oocytes were isolated by manual dissection and stored overnight in MBS-H at 18°C. Five healthy oocytes were frozen in 100 μl of immunoprecipitation buffer (see above) at approximately $GVBD_{50}$. Samples were later thawed, lysed, and centrifuged, and the clear supernatant was divided into two aliquots for anti-TAG- and anti-*cdc2*-associated histone H1 kinase assays.

Lysates were first precleared with protein A-Sepharose. Samples were then immunoprecipitated with the appropriate antiserum. First, 1 μl of anti-TAG or anti-*cdc2* serum was added to each sample and left on ice for 30 min. Next, protein A-Sepharose was added, and samples were left on ice for an additional 30 min with occasional mixing. Finally, immune complexes were spun through 1 ml of 10% sucrose–immunoprecipitation buffer, washed three times with 1 ml of immunoprecipitation buffer, and then transferred to a new tube, and any remaining immunoprecipitation buffer was removed before the addition of 16 μl of 1× H1 kinase buffer (20 mM HEPES [pH 7.4], 30 mM β-mercaptoethanol, 0.1 mg of BSA per ml, 10 mM $MgCl_2$, 1.7 mg of histone H1 per ml, 5 μg of heat-stable protein kinase A inhibitor per ml, 100 μM ATP, 0.25 mCi of [γ-^{32}P]ATP). Following incubation at 30°C for 15 min, reactions were stopped by the addition of 4 μl of 5× sample buffer (1× sample buffer is 50 mM Tris HCl [pH 6.8], 2% SDS, 1 M β-mercaptoethanol, 10% glycerol, and 0.005% bromphenol blue). Samples were boiled for 3 min and analyzed by SDS-15% PAGE and autoradiography.

Nucleotide sequence accession numbers. The nucleotide sequences for clones A and B (Fig. 1) have been assigned GenBank accession numbers M60681 and M60682, respectively.

RESULTS

Mutation of both Thr-14 and Tyr-15 results in an activated p34^{cdc2} in *Xenopus* oocytes. A *Xenopus cdc2* clone (see Materials and Methods) was used as a template for site-directed mutagenesis of putative Thr and Tyr phosphorylation sites. Mutants changing Thr-14 to Ala and Tyr-15 to Phe were made as described in Materials and Methods. These particular mutations were chosen because the dephosphorylation of both of these amino acids is known to be important in the activation of p34^{cdc2} (23, 34, 48, 61). Each of the *cdc2* mutant RNAs was translated in vitro as described in Materials and Methods and found to yield approximately equivalent amounts of protein, as determined by SDS-PAGE and autoradiography (data not shown). As seen previously (13), microinjection of *cdc2* wild-type RNA into resting oocytes had no apparent effect. When the single mutants Thr-14→Ala and Tyr-15→Phe were assayed by microinjection of in vitro-transcribed RNA into *Xenopus* oocytes, the oocytes failed to undergo GVBD (Table 1). In contrast, the double mutant Ala-14/Phe-15 did result in release from metaphase arrest of microinjected oocytes. Therefore, this mutant can bypass some restriction point which is present in the resting *Xenopus* oocyte.

TABLE 1. Biological activity of *cdc2* mutants

RNA[a] or hormone	No. of oocytes	% GVBD	Histone H1 kinase activity[b]
Wild type	40	0	−
Ala-14	27	0	−
Phe-15	27	0	−
Ala-161	12	0	−
Glu-161	22	0	−
Ala-14/Phe-15	67	70	+
Ala-14/Ala-161	24	0	−
Ala-14/Glu-161	24	0	−
Phe-15/Ala-161	24	0	−
Phe-15/Glu-161	24	0	−
Ala-14/Phe-15/Ala-161	24	0	−
Ala-14/Phe-15/Glu-161	24	0	−
Progesterone	91	89	+

[a] Data shown are totals from two to four experiments per mutant. Mutants are designated by the amino acid followed by the position in *Xenopus cdc2*.
[b] Total p34^{cdc2} was immunoprecipitated with carboxy-terminal *cdc2* antiserum and then assayed for histone H1 kinase activity. +, detectable kinase activity; −, lack of detectable histone H1 phosphorylation.

Interestingly, the double mutant Ala-14/Phe-15 consistently induced oocyte maturation at a rate that was much slower than that in response to progesterone (Fig. 2). While progesterone-treated oocytes reached $GVBD_{50}$ in approximately 6 h, the oocytes injected with the mutant RNA required approximately 12 h. The double mutant Ala-14/Phe-15 was also less efficient at inducing maturation, resulting in less than $GVBD_{50}$ after 20 h in some experiments. This result may be due to decreased survival of the oocytes during the longer time course of these experiments. The long maturation times observed for the double mutant Ala-14/Phe-15 might be explained by an absence of free cyclin available to bind to the newly translated p34^{cdc2}. We addressed this question in the experiments described below by coinjection of RNAs encoding mutant p34^{cdc2} and cyclin B1.

Two p34^{cdc2} mutants at Thr-161 were also constructed (see Materials and Methods). This amino acid rests in a region conserved among protein kinases. The analogous amino acid in the catalytic subunit of cyclic AMP (cAMP)-dependent protein kinase is Thr-241 (39, 40). Mutation of Thr-241 to Ala results in decreased binding to the regulatory subunit and increased kinase activity (39, 40). The p34^{cdc2} mutant Thr-161→Ala was first examined for its ability to

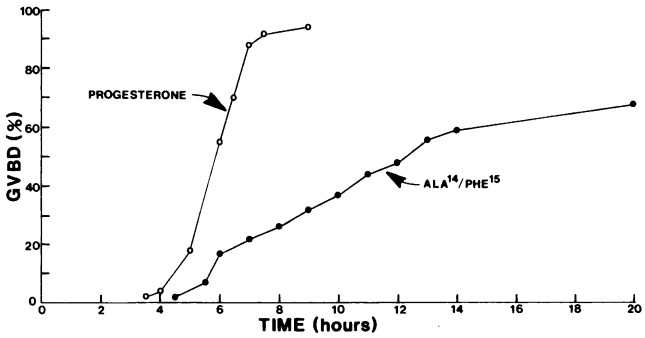

FIG. 2. Graph showing time course of the Ala-14/Phe-15 mutant. Stage VI oocytes were microinjected with 50 nl of a 3-mg/ml solution of p34^{cdc2} Ala-14/Phe-15 mutant RNA (●) or treated with 30 μM progesterone (○). Oocytes were scored for GVBD as described in Materials and Methods.

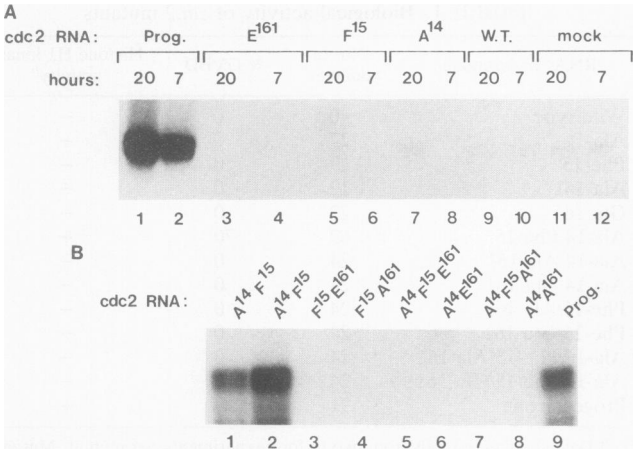

FIG. 3. Histone H1 kinase assays. (A) Single mutants. Oocytes were microinjected with wild-type or mutant *cdc2* RNA as described in Materials and Methods. At the times indicated, oocytes were lysed and frozen in immunoprecipitation buffer and then thawed and immunoprecipitated with carboxy-terminal *cdc2* antisera at a later time. Even-numbered lanes are from oocytes isolated at 7 h postinjection, and odd-numbered lanes are from oocytes isolated at 20 h postinjection. Lanes 1 and 2 are from progesterone-treated oocytes (Prog.), and lanes 3 through 10 are oocytes injected with the following RNAs: lanes 3 and 4, Glu-161 mutant; lanes 5 and 6, Phe-15 mutant; lanes 7 and 8, Ala-14 mutant; lanes 9 and 10, wild-type *cdc2*. Lanes 11 and 12 are from untreated oocytes. Each lane represents one oocyte. The autoradiograph was exposed for 12 h at −70°C with an intensifying screen. (B) Double and triple mutants. Oocytes were treated as described above except that only a 20-h time point was taken. Lanes: 1 and 2, Ala-14/Phe-15 (two independent isolates); 3, Phe-15/Glu-161 mutant; 4, Phe-15/Ala-161 mutant; 5, Ala-14/Phe-15/Glu-161 mutant; 6, Ala-14/Glu-161 mutant; 7, Ala-14/Phe-15/Ala-161 mutant; 8, Ala-14/Ala-161 mutant; 9, progesterone. The autoradiograph was exposed for 18 h at −70°C with an intensifying screen. Each lane represents one oocyte.

induce oocyte maturation. This mutant showed no effect upon microinjection into *Xenopus* oocytes. This was also true for both of the *cdc2* double mutants, Ala-14/Ala-161 and Phe-15/Ala-161, and the triple mutant, Ala-14/Phe-15/Ala-161. These results support current data suggesting a regulatory role for the phosphorylation of Thr-161 of $p34^{cdc2}$ (2, 5, 24, 33, 61). In addition to the *cdc2* mutant Thr-161→Ala, we also constructed the mutant Thr-161→Glu. As Thr-161 is phosphorylated in active $p34^{cdc2}$, we reasoned that mutation to Glu may simulate an active protein because it may mimic a phosphorylated Thr (39, 40). Microinjection of in vitro-transcribed *cdc2* mutant Thr-161→Glu RNA into oocytes did not result in GVBD. *cdc2* double mutants containing Glu-161 and either Ala-14 or Phe-15 failed to result in GVBD as did the triple mutant, Ala-14/Phe-15/Glu-161. These data are summarized in Table 1.

All mutants were also assayed for their ability to activate MPF following microinjection of synthetic RNAs, by assaying phosphorylation of histone H1 in an immune complex kinase assay (Fig. 3; Table 1). Only the Ala-14/Phe-15 double mutant induced a level of kinase activity comparable to that obtained from progesterone-treated oocytes. This finding suggests that the *cdc2* Ala-14/Phe-15 mutant protein can either form an active MPF complex or induce activation of pre-MPF. We describe below experiments demonstrating that this mutant protein can be recovered in an active complex which phosphorylates histone H1 in vitro.

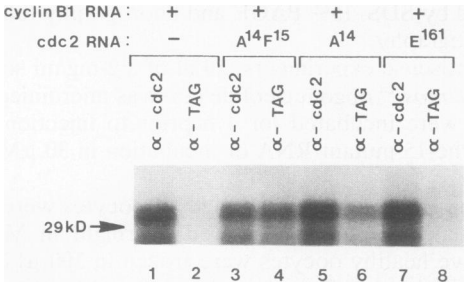

FIG. 4. Histone H1 kinase assays of *cdc2* mutants. Oocytes were microinjected with a solution of 100 ng of wild-type or mutant *cdc2* RNA and 100 ng of cyclin B1 RNA. At approximately $GVBD_{50}$, oocytes were lysed and frozen. Lysates were split into two equal aliquots for immunoprecipitation by either anti-TAG (α-TAG) or anti-*cdc2* (α-*cdc2*) serum. Following immunoprecipitation, samples were assayed for histone H1 kinase activity as described in Materials and Methods. Odd-numbered lanes are from anti-*cdc2* immunoprecipitates; even-numbered lanes are from anti-TAG immunoprecipitates. Oocytes were injected with cyclin B1 RNA and glass-distilled water (lanes 1 and 2), Ala-14/Phe-15 mutant RNA (lanes 3 and 4), Ala-14 mutant RNA (lanes 5 and 6), or Glu-161 mutant RNA (lanes 7 and 8).

Ability of $p34^{cdc2}$ mutants to bind to cyclin B1. To distinguish the biochemical activity of these $p34^{cdc2}$ mutants from the endogenous $p34^{cdc2}$, it was necessary to attach a peptide TAG sequence that could function as a specific epitope for immunoprecipitation. The TAG sequence was attached to the carboxy terminus of $p34^{cdc2}$ by using an oligonucleotide encoding the 13-amino-acid TAG sequence (see Materials and Methods for details). In the construction of the *cdc2*-TAG clone, two carboxy-terminal amino acids of $p34^{cdc2}$ were also mutated. These mutations are Arg-301→Lys-301 and Gln-302→Ile-302. These changes were found to have no significant effect on in vitro kinase activity or induction of oocyte maturation (data not shown).

In vitro-translated *cdc2*-TAG proteins were immunoprecipitated with either carboxy-terminal anti-*cdc2* or anti-TAG serum, and it was found that only anti-TAG serum was capable of recognizing the *cdc2*-TAG proteins (data not shown). Importantly, the carboxy-terminal anti-*cdc2* serum does not recognize the *cdc2*-TAG proteins.

The TAG antiserum was first used to examine the histone H1 kinase activity associated with the different *cdc2* mutants. Oocytes were coinjected with RNAs encoding both cyclin B1 and *cdc2*, after which they were lysed at $GVBD_{50}$. As a control to show specificity of the anti-TAG serum, cyclin B1 was injected alone and immunoprecipitated with anti-TAG or anti-*cdc2* serum. In this control experiment, only the anti-*cdc2* immunoprecipitate showed detectable histone H1 kinase activity (Fig. 4, lanes 1 and 2). The anti-TAG and anti-*cdc2* immunoprecipitates from oocytes expressing Ala-14–TAG or Ala-14/Phe-15–TAG together with cyclin B1 exhibited comparable histone H1 kinase activities (Fig. 4, lanes 3 to 6). The difference in H1 kinase activity between the Ala-14 *cdc2* mutant immunoprecipitate and the Ala-14/Phe-15 immunoprecipitate is likely due to the fact that the Ala-14 *cdc2* mutant protein can also be phosphorylated on Tyr-15. Therefore, only a percentage of the total Ala-14 *cdc2* protein is likely to be active. However, anti-TAG immunoprecipitates prepared from oocytes expressing Glu-161–TAG and cyclin B1 were negative for associated H1 kinase activity (Fig. 4, lanes 7 and 8).

To compare the abilities of the *cdc2* mutant proteins to

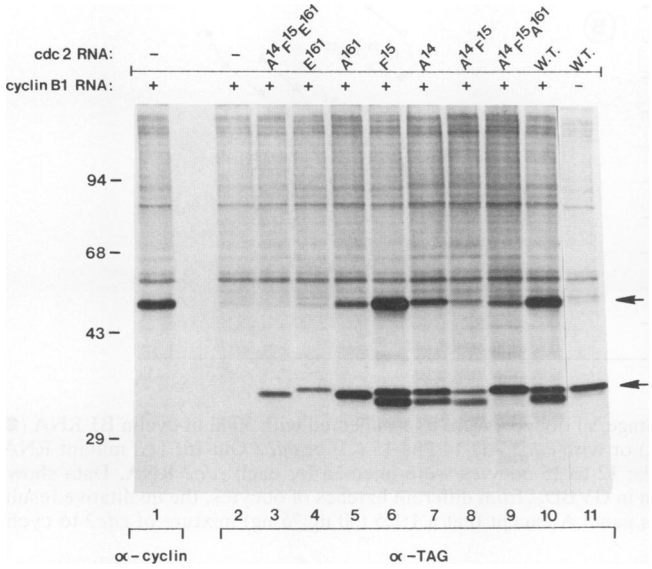

FIG. 5. Coimmunoprecipitation of cyclin B1 with p34^{cdc2} mutants. Stage VI oocytes were microinjected with a mixture of cyclin B1 RNA and either mutant or wild-type *cdc2* RNA. Oocytes were labeled for 3 to 4 h in MBS-H containing 0.5 mCi each of [^{35}S]Cys and [^{35}S]Met per ml. Immunoprecipitations were done as described in Materials and Methods. Lane 1 is from oocytes microinjected with cyclin B1 and immunoprecipitated with anti-cyclin B1 serum (13). Samples in lanes 2 through 11 were immunoprecipitated with anti-TAG serum. Lane 11 is from oocytes microinjected with *cdc2* RNA alone and lysed at 3.5 h postinjection (no GVBD occurred). Lanes 2 through 10 are from oocytes coinjected with both cyclin B1 and *cdc2* RNAs as follows: lane 2, glass-distilled water; lane 3, Ala-14/Phe-15/Glu-161; lane 4, Glu-161; lane 5, Ala-161; lane 6, Phe-15; lane 7, Ala-14; lane 8, Ala-14/Phe-15; lane 9, Ala-14/Phe-15/Ala-161; lane 10, wild type. Samples were subjected to SDS–15% PAGE and then to fluorography and autoradiography. Each lane represents four oocytes. The exposure time was 7 days. The upper arrow indicates the position of cyclin B1, and the lower arrow indicates the position of *cdc2*. Molecular size standards are indicated in kilodaltons at the left.

bind cyclin B1, we carried out experiments in which mutant *cdc2*-TAG proteins were coexpressed with cyclin B1 in microinjected oocytes and radiolabeled proteins were immunoprecipitated with anti-TAG serum. The oocytes were metabolically labeled until they reached $GVBD_{50}$, lysed and immunoprecipitated with anti-TAG serum, and analyzed by SDS–15% PAGE (Fig. 5). Several oocytes were also injected with either wild-type *cdc2*-TAG RNA or cyclin B1 RNA alone and metabolically labeled with ^{35}S for 3.5 h for the wild-type oocytes (which did not undergo GVBD) and until $GVBD_{50}$ for the cyclin B1-injected oocytes. The *cdc2*-TAG-injected oocytes were then immunoprecipitated with anti-TAG serum (Fig. 5, lane 11), and the cyclin B1 injected oocytes were immunoprecipitated with anti-cyclin B1 serum (Fig. 5, lane 1). The variability observed in recovery of labeled p34^{cdc2} proteins likely reflects variable recovery through the immunoprecipitation procedure. In support of this view, the amounts of the Glu-161 and Ala-14/Phe-15/Glu-161 *cdc2* proteins, which appear lower than the amount of the wild-type *cdc2* in this autoradiogram, appear at higher than wild-type levels in other experiments (data not shown). However, for any given *cdc2* mutant, the ratio of coimmunoprecipitated ^{35}S-labeled cyclin B1 to the amount of ^{35}S-labeled *cdc2* was reproducible in multiple experiments. Therefore, autoradiographs from five independent experiments were analyzed by densitometry to compare the relative amounts of cyclin B1 and p34^{cdc2} in each lane. The doublets seen in lanes 6, 7, 8, and 10 of Fig. 5 are probably due to differences in the level of phosphorylation of p34^{cdc2}, but this matter was not investigated. If the ratio of coimmunoprecipitated cyclin to wild-type *cdc2*-TAG is taken as 100%, then the average values from five independent experiments (determined by densitometry) were as follows for the ratio of coimmunoprecipitated cyclin to mutant *cdc2*-TAG: Ala-14, 41%; Phe-15, 41%; Ala-14/Phe-15, 67%; Ala-161, 25%; Glu-161, 21%; Ala-14/Phe-15/Ala-161, 27%; and Ala-14/Phe-15/Glu-161, 33%. This can be seen in Fig. 5, in which Ala-14 (lane 7), Phe-15 (lane 6), Ala-14/Phe-15 (lane 8), and the wild type (lane 10) show higher levels of cyclin binding than do Ala-161 (lane 5), Glu-161 (lane 4), and the two triple mutants, Ala-14/Phe-15/Ala-161 (lane 9) and Ala-14/Phe-15/Glu-161 (lane 3). These results indicate that phosphorylation of Thr-161 is significant in binding of p34^{cdc2} to cyclin B1 in *Xenopus* oocytes.

Kinetics of maturation of oocytes coexpressing cyclin B1 and *cdc2* mutants. In observing oocytes coexpressing cyclin B1 and each of the various *cdc2* mutants, it was found that some of the *cdc2* mutants altered the kinetics of oocyte maturation in response to expression of cyclin B1. In addition, this effect was dependent on the ratio of cyclin B1 to *cdc2* RNA injected. Oocytes microinjected with a 1:2 ratio of *cdc2* Ala-14/Phe-15 mutant RNA and cyclin B1 RNA matured at about the same rate as did those injected with cyclin B1 alone (Fig. 6A). In contrast, coinjection of *cdc2* wild type or *cdc2* mutant Glu-161 and cyclin B1 RNA resulted in slower induction of maturation than injection of cyclin B1 RNA alone (Fig. 6A). These findings on the rate of oocyte maturation can be summarized as follows: Ala-14/Phe-15 > wild type > Glu-161 (upon coinjection with cyclin B1 RNA). The other *cdc2* mutant RNAs also induced a slower rate of maturation when coinjected with cyclin B1 RNA than did cyclin B1 injected alone (data not shown).

When oocytes were microinjected with a 1:1.5 ratio of *cdc2* and cyclin B1 RNAs (using less cyclin B1 RNA but the same amount of *cdc2* RNA), the oocytes matured more slowly overall and differences between the different *cdc2* mutants became more striking (Fig. 6B). The Ala-14/Phe-15 mutant induced GVBD more rapidly than did cyclin B1 alone. The wild-type *cdc2* RNA coinjected with cyclin B1 induced maturation more slowly than did the Ala-14/Phe-15 mutant coinjected with cyclin B1 but more rapidly than did cyclin B1 alone (Fig. 6B). The Glu-161 mutant RNA when coinjected with cyclin B1 induced maturation at a rate much slower than that induced by cyclin B1 RNA injected alone (Fig. 6B). Once again, these results can be summarized as follows: Ala-14/Phe-15 > wild type > Glu-161. The other mutants can be classified into two groups based on the results obtained upon coinjection with cyclin B1 RNA. The first group induced maturation at a rate similar to the wild-type *cdc2* rate and included Ala-14, Phe-15, and Ala-14/Phe-15/Glu-161 (data not shown). The second group includes Ala-161 and Ala-14/Phe-15/Ala-161 and induced maturation upon coinjection at a rate between that of cyclin B1 alone and that of cyclin B1 coinjected with Glu-161 (data not shown).

Progesterone-induced maturation is not affected by *cdc2* mutants. In contrast to the results obtained with cyclin B1 coinjected with the *cdc2* mutants, incubation of the oocytes in progesterone following injection showed little or no effect

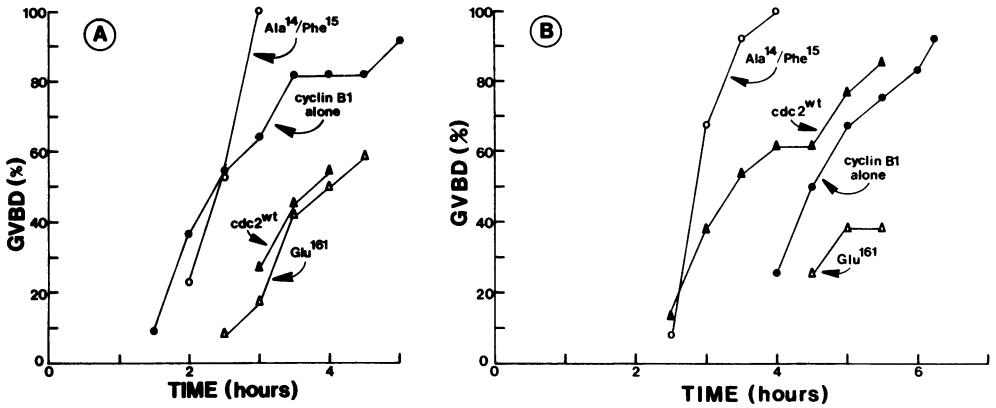

FIG. 6. Effect of coinjection of p34^{cdc2} mutants with cyclin B1. (A) Stage VI oocytes were microinjected with 50 nl of cyclin B1 RNA (●) or a 1:2 (50 ng:100 ng) mixture of wild-type *cdc2* to cyclin B1 RNA (▲) or with *cdc2* Ala-14/Phe-15 (○) or *cdc2* Glu-161 (△) mutant RNA. Oocytes were scored for GVBD as described in Materials and Methods; 12 to 15 oocytes were injected for each *cdc2* RNA. Data shown represent only one experiment. Although there was some variability seen in $GVBD_{50}$ from different batches of oocytes, the qualitative results obtained were consistent from one experiment to the next. (B) Same as panel A except that a 1:1.5 (50 ng:75 ng) mixture of *cdc2* to cyclin B1 RNA was used.

on the rate of maturation. As shown in Fig. 7, even mutants which had exhibited altered rates of oocyte maturation in coinjection experiments with cyclin B1 RNA, such as Ala-14/Phe-15 and Glu-161, exhibited oocyte maturation at approximately the same rate as did those treated with progesterone alone. This finding may be due to the different modes of induction of maturation. Progesterone-induced maturation involves a protein synthesis requirement, although new cyclin synthesis is not required (42, 46). In contrast, induction of GVBD by cyclin B1 protein does not require protein synthesis (53).

Effect of *mos*^{Xe} RNA and antisense oligonucleotides on p34^{cdc2} mutants. *cdc2* mutant genes were assayed to determine whether they could alter the rate of c-*mos*-induced maturation. *cdc2* wild-type or mutant RNAs and *mos*^{Xe} RNAs were coinjected into resting *Xenopus* oocytes. A slight change in the rate of maturation was seen when *cdc2* mutant or wild-type RNA was injected in combination with *mos*^{Xe} RNA (Fig. 8). Both the Ala-14/Phe-15 and Ala-14/Phe-15/Glu-161 mutants showed a slightly increased rate of maturation, $GVBD_{50}$ being approximately 4.5 h for coinjection versus 5.5 h for *mos* alone. In contrast, the *cdc2* single mutants Ala-161 and Glu-161, when coinjected with *mos* RNA, showed slightly decreased rates of maturation, similar to that obtained with coinjection of *mos* and *cdc2* wild-type RNA (Fig. 8 and data not shown).

We also assayed the effect of microinjecting antisense oligonucleotides to *mos*^{Xe} RNA on the induction of GVBD by the activated *cdc2* Ala-14/Phe-15 mutant. *mos* protein is required for progesterone-induced maturation; therefore, progesterone was used as a control (57). Stage VI oocytes were collected and either injected with antisense *mos*^{Xe} oligonucleotides or left in buffer. After 4 h, half of the antisense oligonucleotide-injected oocytes were injected with the *cdc2* Ala-14/Phe-15 mutant RNA. The remaining antisense oligonucleotide-injected oocytes were treated with progesterone. At approximately the same time, the uninjected oocytes were either treated with progesterone or

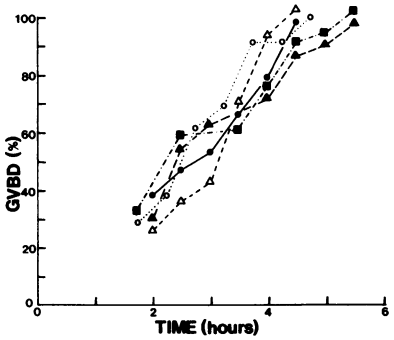

FIG. 7. Effect of p34^{cdc2} mutant RNAs on progesterone-induced maturation. Stage VI oocytes were microinjected with 50 nl of a 2-mg/ml solution of wild type *cdc2* RNA (●) or with *cdc2* Ala-14/Phe-15 mutant RNA (○), *cdc2* Ala-161 mutant RNA (△), *cdc2* Glu-161 mutant RNA (▲), or no RNA (■). Approximately 1 h postinjection, oocytes were transferred to a solution of 30 μM progesterone in MBS-H. Time given is post progesterone incubation for all samples. Oocytes were scored for GVBD as described in Materials and Methods.

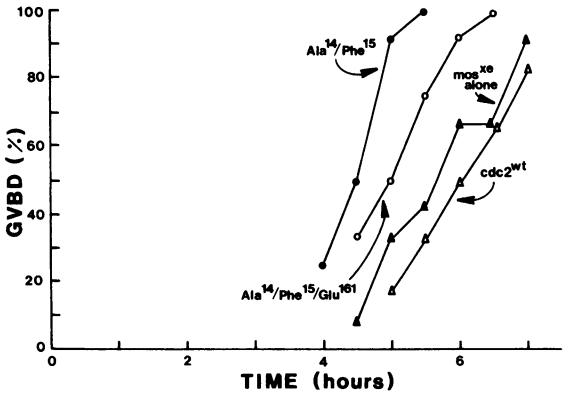

FIG. 8. Effect of coinjection of p34^{cdc2} mutants and *mos*^{Xe} RNA. Oocytes were microinjected with 100 ng of *mos*^{Xe} RNA (▲) or a 1:1 mixture of *mos*^{Xe} RNA and wild-type *cdc2* RNA (△) or with *cdc2* Ala-14/Phe-15 (●) or *cdc2* Ala-14/Phe-15/Glu-161 (○) mutant RNA. Oocytes were scored for GVBD as described in Materials and Methods.

TABLE 2. Effect of antisense *mos* oligonucleotides on induction of GVBD by the Ala-14/Phe-15 p34^{cdc2} mutant[a]

Treatment	Antisense *mos*^{Xe}	% GVBD	No. of oocytes injected
Ala-14/Phe-15 RNA	+	0	32
	−	38	31
Progesterone	+	5	38
	−	95	42

[a] Oocytes were injected with antisense *mos*^{Xe} oligonucleotides; 4 h later, healthy oocytes were treated with progesterone or injected with Ala-14/Phe-15 RNA as described in Materials and Methods. GVBD was assayed at ca. 20 h postinjection and verified by fixation and manual dissection of the oocyte. Data shown are totals from three separate experiments.

injected with *cdc2* Ala-14/Phe-15 mutant RNA. As expected, the progesterone treated oocytes which had been injected with the antisense *mos*^{Xe} oligonucleotide did not mature, while the control oocytes matured (Table 2). As seen previously (Table 1), the *cdc2* double mutant-injected oocytes matured less efficiently than did those treated with progesterone. However, none of the antisense oligonucleotide-injected oocytes reached GVBD. In addition, the injection of a control oligonucleotide prior to injection of the *cdc2* Ala-14/Phe-15 mutant RNA had no apparent effect on induction of GVBD (data not shown). These results suggest a role for c-*mos* in *cdc2* Ala-14/Phe-15-induced maturation.

DISCUSSION

Thr-14 and Tyr-15 of p34^{cdc2} are both involved in the regulation of MPF activity in *Xenopus* oocytes. We have shown that both Thr-14 and Tyr-15 in p34^{cdc2} are important in maintaining G_2/M arrest in *Xenopus* oocytes. It has also been demonstrated both in vitro and in mammalian fibroblasts that Thr-14 and Tyr-15 are involved in the regulation of the kinase activity of p34^{cdc2} (5, 34, 48, 61). Biochemical techniques and site-directed mutagenesis were used to determine the significance and location of these phosphorylation events. Gould and Nurse previously identified Tyr-15 in the ATP-binding domain of *S. pombe* p34^{cdc2} as a site of phosphorylation (23). Mutating Tyr-15 to Phe and expressing the mutant protein in a p34^{cdc2}-deficient yeast strain resulted in premature entry into mitosis (23). Biochemical evidence for phosphorylation of Thr-14 of p34^{cdc2} in vertebrates suggests that this may also be a site of regulatory phosphorylation (33). Indeed, the significance of this site has been verified here and elsewhere by site-directed mutagenesis (34, 48, 61). Mutation of p34^{cdc2} Thr-14 to Ala or Tyr-15 to Phe independently did not result in an unregulated p34^{cdc2} upon expression in *Xenopus* oocytes (Table 1) or in fibroblasts (34, 48). Only the *cdc2* Ala-14/Phe-15 double mutant could induce *Xenopus* oocyte maturation (Table 1) and premature mitotic events when expressed in mammalian fibroblasts (34). This finding demonstrates that Thr-14 is also a site of regulatory phosphorylation for p34^{cdc2} in at least some vertebrates. This amino acid, although present in fission yeast cells, is apparently not a regulatory site, as a Phe-15 mutation is sufficient for activation (23). Interestingly, the budding yeast *Saccharomyces cerevisiae* appears to have a unique method of regulation for entry into mitosis which does not involve dephosphorylation of the analogous amino acids, Thr-18 and Tyr-19, in *CDC28* (1, 62).

The p34^{cdc2} double mutant Ala-14/Phe-15 induces a slow rate of oocyte maturation upon microinjection into resting oocytes (Fig. 2). This is the first demonstration of a *cdc2* mutant protein which can bypass the G_2/M arrest point in *Xenopus* oocytes. While it has not been shown unambiguously, it appears that dephosphorylation of both Thr-14 and Tyr-15 on p34^{cdc2} can lead to activation of pre-MPF. Unfortunately, it is difficult to determine whether p34^{cdc2} in active MPF complexes derives from pre-MPF complexes or, alternatively, derives from free p34^{cdc2} which has been newly incorporated into MPF. Nevertheless, in oocytes which have been induced to mature by injection of the Ala-14/Phe-15 mutant *cdc2* RNA, we have demonstrated that both mutant and wild-type p34^{cdc2} can be recovered as active histone H1 kinase complexes (Fig. 4).

Microinjection of the *cdc25* protein into oocytes can result in rapid induction of GVBD (21). It is possible that this is the endogenous mode of activation in oocytes and that this activation is regulated by *cdc25* phosphatase activity. Support for this model is found in the demonstration that several different forms of the human *cdc25* protein can bind to and be activated by cyclin B in vitro (17). A *cdc25* homolog, p72, is present in resting oocytes and associates with a *cdc2*/cyclin B complex in a cell cycle-dependent manner (29). The slow induction of maturation by the Ala-14/Phe-15 mutant is likely due to the need for p34^{cdc2} to bind cyclin protein in order to form an active complex (60). In the oocyte, there is less cyclin protein than p34^{cdc2} (32), and the rate of GVBD is higher when oocytes are coinjected with RNA encoding both the *cdc2* Ala-14/Phe-15 mutant and cyclin B1 than when they are injected with the *cdc2* mutant RNA alone (Fig. 6 versus Fig. 2). Therefore, cyclin availability is likely to be rate limiting in induction of GVBD by the *cdc2* Ala-14/Phe-15 mutant. Immunoprecipitation of the *cdc2* Ala-14/Phe-15–TAG mutant in the absence of coinjected cyclin RNA allows recovery of H1 kinase activity in anti-TAG immunoprecipitates. This finding suggests that this mutant induces GVBD by formation of an active H1 kinase complex. Our data support a model in which oocytes remain arrested prior to maturation by virtue of the phosphorylation of both Thr-14 and Tyr-15 on the p34^{cdc2} present in pre-MPF. The activating event for pre-MPF would thus be dephosphorylation of these residues.

Induction of GVBD by the p34^{cdc2} Ala-14/Phe-15 mutant is intriguing for a number of reasons. Oocytes are unique in their regulation of MPF, as seen by the fact that *cdc2* is present in an inactive pre-MPF complex in resting oocytes (18, 32). Transient expression of the *cdc2* Ala-14/Phe-15 mutant under the control of a strong promoter in HeLa cells resulted in nuclear envelope breakdown and chromosome condensation, but it did not result in mitotic spindle formation, suggesting the need for other factors in mitotic regulation (34). It has not yet been determined which mitotic events, other than nuclear envelope breakdown, have occurred in oocytes injected with the *cdc2* Ala-14/Phe-15 mutant. Despite the inability of the Ala-14/Phe-15 to drive fibroblasts directly into mitosis, it was found to give rise to an increase in in vitro histone H1 kinase activity (34). However, formation of the active kinase was found to depend on additional phosphorylation as well as cyclin binding (34, 48, 61).

There is currently evidence for several methods of regulation of *cdc2* activity by phosphorylation. The first discovered was Tyr-15 dephosphorylation in *S. pombe*, as dis-

cussed previously (23). Another is shown in this study: in *Xenopus* oocytes, both Thr-14 and Tyr-15 are involved in regulation of activity. Similarly, in somatic cells of vertebrates, both Thr-14 and Tyr-15 of *cdc2* are involved in regulation of *cdc2* activity (34). Interestingly, in somatic cells, the dephosphorylation of Thr-14 and Tyr-15 appears to be linked to the completion of DNA synthesis (34), while in oocytes, dephosphorylation of these amino acids is triggered by an external signal such as progesterone (this report; 12, 30). A third method of regulation may have been found in *S. cerevisiae* (1, 62). Although the *S. cerevisiae CDC28* protein is phosphorylated on Tyr-19 in a cell cycle-dependent manner (1, 62), mutation of both Thr-18 to Ala and Tyr-19 to Phe of *CDC28* does not lead to deregulation of entry into mitosis (1, 62).

A role for p39^{mos} in the activation of MPF. What is the significance of c-*mos*^{Xe} protein synthesis on the induction of GVBD by the *cdc2* Ala-14/Phe-15 double mutant? Expression of the *mos* protein has been detected only in vertebrate cells undergoing meiotic maturation (14, 64). It has also been shown that the kinase activity of p39^{mos} is necessary for its function in induction of meiotic maturation, as a kinase-inactive p39^{mos} cannot induce GVBD alone (13, 16). In contrast to the wild-type *mos* protein, the kinase-inactive p39^{mos} is also not capable of increasing the rate of oocyte maturation upon coinjection with cyclin B1 or B2 RNA (13, 16). The expression of the *mos* protein could trigger release from G_2/M arrest in resting oocytes by phosphorylation of a specific protein or proteins. There are many proteins involved in meiotic maturation, any one of which could represent a target for *mos* kinase activity. One previously proposed function for *mos* is a role in the activation and stabilization of cyclin (27). This hypothesis was based on the identification of *mos* as CSF, an activity which is presumed to stabilize MPF (27, 58). In support of this model, c-*mos* has been shown to play a role in the stablization of cyclin B in mouse eggs (49). Furthermore, *mos* can accelerate cyclin-induced maturation when coinjected into *Xenopus* oocytes (13), and *mos* reportedly phosphorylates cyclin B2 in an in vitro kinase assay (53). Coinjection of *mos* and cyclin B1 or B2 RNA, however, does not lead to increased cyclin phosphorylation in vivo (13). In addition, site-directed mutagenesis has been used to show that cyclin B2 phosphorylation by p34^{cdc2} or microtubule-associated protein kinase is not required for induction of maturation (28). These results suggest that the induction of maturation by *mos* may be by a pathway other than *mos* phosphorylation of cyclin.

Another possible function for *mos* in the induction of GVBD is phosphorylation of tubulin (66). *mos*^{Xe} can associate with and phosphorylate tubulin in vivo and in vitro (66). In addition, p39^{mos} is specifically localized to the mitotic spindle in *mos*^{Xe}-transformed NIH 3T3 cells, suggesting a role in spindle formation (66). As MPF activation precedes spindle formation, it is unlikely that phosphorylation of tubulin is the role for *mos* in MPF activation (26). Phosphorylation of tubulin, however, can play a role in tubulin polymerization (63), and localization of *mos* to the mitotic spindle is likely to be significant in maintenance of metaphase arrest by *mos*.

A third possible role for *mos* is activation of p34^{cdc2} by regulating the phosphorylation and dephosphorylation of Thr-161. It has been shown that Thr-161 phosphorylation is required for formation of an active histone H1 kinase (this report; 5, 24, 61). If *mos* expression regulates the phosphorylation and dephosphorylation of Thr-161, this may lead to MPF activation and thus to GVBD. Evidence has been reported recently for an activity present in *Xenopus* eggs which phosphorylates p34^{cdc2} on Thr-161 (61). In addition, an activity known as INH, a form of protein phosphatase 2A isolated from *Xenopus* oocytes, has been reported to dephosphorylate Thr-161 in vitro (38). Either of these enzymatic activities is a reasonable candidate for a substrate of the p39^{mos} kinase. It should also be noted that our data do not rule out the possibility that *mos* acts to activate pre-MPF present in the oocyte by regulation of Thr-14 and Tyr-15 dephosphorylation.

p34^{cdc2} Thr-161 phosphorylation and cyclin B1 binding. Thr-161 plays a role in the activation of p34^{cdc2} in *X. laevis*, as well as other eukaryotes (Thr-161 is analogous to Thr-167 in *S. pombe*). Binding of p34^{cdc2} phosphorylation site mutants to cyclin has recently been assayed in a number of different systems, with generally consistent results. We have shown here that when Thr-161 in *Xenopus* p34^{cdc2} was mutated to Ala, there was a drastic decrease in the amount of cyclin B1 which could be coimmunoprecipitated when both were expressed in *Xenopus* oocytes (Fig. 5). Similarly, mutation of Thr-167 to Ala in *S. pombe* p34^{cdc2} resulted in a decreased binding to p56^{cdc13}, a cyclin B1 homolog (24), and a Thr-167→Val mutation in *S. pombe cdc2* could not complement a *cdc2*-deficient strain (2). These results indicate the requirement for a phosphorylatable amino acid at position 161 in *Xenopus cdc2* and position 167 in *S. pombe cdc2* (2, 24). Ducommun and coworkers found that a Thr-161→Ala mutant in human p34^{cdc2} also could not bind to either cyclin A or B in an in vitro system (5). Solomon and coworkers, however, did not see any significant decrease in cyclin binding to *Xenopus* p34^{cdc2} for the Thr-161→Ala mutant (61). Interestingly, both Ducommun et al. and Solomon et al. used vertebrate *cdc2* genes and similar assay conditions (5, 61). This apparent contradiction is probably due to the details of the experimental conditions used. It was also found that a Ser at position 161 in *Xenopus* and human p34^{cdc2} or 167 in *S. pombe* p34^{cdc2} could give rise to an active p34^{cdc2} complex (24, 61). In conclusion, phosphorylation of Thr-161 of *Xenopus* and human p34^{cdc2} and Thr-167 of *S. pombe* p34^{cdc2} is likely to be required for formation of a stable p34^{cdc2}/cyclin complex in vivo and in vitro (this report; 2, 5, 24).

Work from other laboratories has demonstrated that some *cdc2* mutants, for example, a human Val-161 *cdc2* mutant (5) or a yeast Ala-167 *cdc2* mutant (24), can induce a dominant negative or lethal phenotype when expressed at high levels in yeast cells. This finding suggests that these mutants may functionally interact with the cell cycle machinery. In the experiments reported here, expression of the Ala-161 p34^{cdc2} mutant had no apparent effect on the induction of GVBD by progesterone (Fig. 7). One potential explanation for this difference is that *Xenopus* oocytes contain a large amount of endogenous *cdc2* protein (approximately 5 ng [32]). In addition, they contain a limited amount of cyclin B protein, the majority of which is apparently in a pre-MPF complex (32). Therefore, the amount of *cdc2* protein synthesized by our mutant RNA is likely insufficient to exhibit dominant inhibition of pre-MPF activation in response to progesterone.

The catalytic subunit of cAMP-dependent protein kinase contains a Thr at a position homologous to that of Thr-161 in *Xenopus* p34^{cdc2} (39, 40). This Thr, Thr-241, appears to be significant for binding to the regulatory subunit of the cAMP-dependent protein kinase (39, 40). Mutation of Thr-241 in vitro to Asp or Glu resulted in mutant proteins that

were still capable of binding to the regulatory subunit (40). In the experiments presented here, mutation of Thr-161 to Glu in *Xenopus* p34^{cdc2} results in a protein product which shows a decreased affinity for cyclin B1 in coimmunoprecipitation (Fig. 5). In addition, this p34^{cdc2} mutant cannot phosphorylate histone H1 in an immune complex kinase assay (Fig. 4). Thus, Glu may not sufficiently imitate a phosphorylated Thr for efficient binding to cyclin B1. However, an interesting result occurred when RNA encoding the *cdc2* Glu-161 mutant was coinjected with cyclin B1 RNA into oocytes. The time required for $GVBD_{50}$ increased, and fewer of the oocytes matured (Fig. 6). Similarly, it has been shown that a Glu-167 mutant of *S. pombe* p34^{cdc2} can rescue a *cdc2*-deficient strain to some degree, although this mutant cannot phosphorylate histone H1 in an in vitro kinase assay, nor can it bind in vitro to cyclin (5, 24). This mutant displays an interesting phenotype when expressed in *S. pombe*, including a high number of mitotic spindles, a delay in exiting mitosis, and an unequal distribution of DNA to daughter cells (5, 24). One may ask why a *cdc2* mutant that cannot bind to cyclin would affect the ability of cyclin to induce maturation. Although we were not able to detect an interaction in vitro, we would propose that in vivo, the p34^{cdc2} Glu-161 mutants in *X. laevis* and humans and the Glu-167 mutant in *S. pombe* may bind to cellular components, such as cyclin and *cdc25*, and exhibit some kinase activity (this report; 5, 21, 24). Interestingly, while the *cdc2* Ala-14/Phe-15/Ala-161 and the Ala-161 *cdc2* mutants both have little or no effect on cyclin-induced maturation, the Ala-14/Phe-15/Glu-161 mutant has the opposite effect of the Glu-161 *cdc2* mutant (that is, the Ala-14/Phe-15/Glu-161 mutant increases the rate of cyclin-induced maturation, while the Glu-161 mutant decreases the rate of cyclin-induced maturation). The reason for this variability is likely to be due to differences in the abilities of these mutant proteins to bind to other cellular factors.

It is possible that the requirement for *mos* in induction of GVBD by the p34^{cdc2} Ala-14/Phe-15 mutant may be explained by an effect on the phosphorylation state of Thr-161. One possible model would have INH present in the resting oocyte to keep the pre-MPF complex inactive. Upon stimulation by progesterone, the oocyte begins synthesis of the *mos* protein, which either directly phosphorylates Thr-161 on *cdc2* or, more likely, activates some other protein which does so. There is currently no evidence for *mos* phosphorylation of p34^{cdc2}, although this does not rule out the possibility of its occurrence. An interesting aspect to this is the requirement of the *cdc2* Ala-14/Phe-15 mutant to induce protein synthesis, as *mos* is not present in the resting oocyte (56). It is possible that proteins other than *mos* also need to be synthesized for induction of GVBD by the *cdc2* Ala-14/Phe-15 double mutant. The *mos* protein does not require the synthesis of other proteins for induction of GVBD, although it does require protein synthesis for entry into meiosis II (65). It has been shown that while MPF can induce GVBD in the absence of protein synthesis (42), it takes a longer period of time to induce GVBD by MPF in the presence of antisense *mos* oligonucleotides than it does in the presence of protein synthesis inhibitors (4). Clearly, induction of oocyte maturation requires numerous interacting factors, only some of which have been characterized. Further investigation of the activation of both MPF and *mos* may lead to the answers to some of these questions.

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