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Phosphorylation of MCM4 by cdc2 protein kinase inhibits the activity of the minichromosome maintenance complex

(DNA replication/cell cycle/protein kinases/*Xenopus*)

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ABSTRACT **In eukaryotes, tight regulatory mechanisms ensure the ordered progression through the cell cycle phases. The mechanisms that prevent chromosomal DNA replication from taking place more than once each cell cycle are thought to involve the function of proteins of the minichromosome maintenance (MCM) family. Here, we demonstrate that *Xenopus* MCM4, a member of the MCM protein family related to Spcdc21/ScCDC54, is part of a large protein complex comprising several other MCM proteins. MCM4 undergoes cell cycle-dependent phosphorylation both in cleaving embryos and in cell-free extracts. MCM4 phosphorylation starts concomitantly with the clearing of the MCM complex from the chromatin during S phase. Phosphorylation is carried out by cdc2/cyclinB protein kinase, which phosphorylates MCM4 *in vitro* at identical sites as the ones phosphorylated *in vivo*. Phosphorylation is specific for cdc2 protein kinase since MCM4 is not a substrate for other members of the cdk family. Furthermore, phosphorylation of MCM4 dramatically reduces its affinity for the chromatin. We propose that the cell cycle-dependent phosphorylation of MCM4 is a mechanism which inactivates the MCM complex from late S phase through mitosis, thus preventing illegitimate DNA replication during that period of the cell cycle.**

Two highly regulated events of the eukaryotic cell cycle are the duplication of the genetic material during chromosomal DNA replication and the segregation of this material at mitosis. It is critical to regulate the timing, the order and the coordination of these events and to ensure the fidelity of these processes so that identical pools of genetic material are distributed to the daughter cells. A critical aspect of cell cycle regulation is that DNA should be replicated in totality once and only once per cell cycle. Solving this problem is achieved despite the presence of multiple origins of replication firing at different times throughout S phase.

Work using *Xenopus* extracts (1) defined the concept of replication licensing factor as a biological activity necessary for DNA replication and that restricts DNA replication to once per cell cycle. These experiments demonstrated that chromosomal DNA, after assembling into nuclear structures in *Xenopus* cell-free extract, will replicate semiconservatively only once even when the assembled nuclei were transferred to a fresh extract. This block to re-replication could be overcome by passage through mitosis or by making the nuclear membrane permeable with nonionic detergent. These observations suggested the existence of one or several factors essential for replication, either recruited within the nucleus at the end of mitosis, when the nuclear membrane reassembles, or that can cross the nuclear membrane of a G_1 nucleus. In either case, the factor is inactivated during S phase once DNA replication has been initiated. Additional studies using G_2-like *Xenopus* extracts (2, 3) further showed that the replication licensing factor was providing an instructive signal toward initiation of DNA replication.

In the yeast *Saccharomyces cerevisiae*, a family of genes showing some of the expected behaviors of the licensing factor was discovered using two different genetic approaches. Firstly, cdc45 and cdc54 were isolated as cold sensitive cdc mutants that showed a G_1/S arrest and were both suppressed in an allele-specific manner by mutation in cdc46 (4). Independently, genes that are essential to support the replication of plasmids carrying a single replication origin were isolated (5, 6). The minichromosome maintenance (MCM) mutants were isolated in this way and fell into three different complementation groups: MCM2, MCM3, and MCM5. MCM5 was later shown to be allelic for cdc46. In addition, two MCM genes (MCM5/Cdc46 and MCM7/Cdc47) have been shown to display cell cycle dependent nuclear localization. Both proteins enter the nucleus at the end of mitosis and disappear once replication has been initiated in S phase, displaying the behavior predicted for a licensing factor protein (7). The MCM2, MCM3, and MCM5/cdc46 predicted polypeptide sequences showed striking similarity to each other, demonstrating their belonging to the same family of proteins (8). Recently, cdc54 has been shown to be a member of this family as well (9). Homologs of MCM proteins have been isolated in fission yeast (10), flies (11), frogs (12–14), and mammals (15) and fall into 6 subfamilies named MCM2 to MCM7.

The putative link between MCMs and licensing factor was recently substantiated by several groups who demonstrated the involvement of at least one MCM protein (MCM3) in licensing activity as originally defined in *Xenopus* extracts. In one method, using complementation of G_2-like extracts defective in replication licensing factor, two groups (12, 14) showed that a fraction from interphase *Xenopus* extracts containing at least the *Xenopus* MCM3 homolog was able to support licensing activity, as defined by the ability to initiate DNA replication in G_2-like extracts. Another approach showed that permeabilized G_2 nuclei were not able to replicate in *Xenopus* extracts previously depleted of the MCM3-containing protein complex, although, the depleted extracts still supported replication of G_1 nuclei (13). These papers provide the first partial molecular characterization of licensing factor. However, the MCM3 containing complex does not fulfill all the predictions of the licensing factor model. MCM3 can cross the intact nuclear membrane of a G_1 nucleus (13, 16). Moreover, these data do not explain how re-replication is normally prevented, a critical property of DNA replication.

The question of primary importance to understand is how replication is integrated with the other cell cycle events and

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Abbreviations: MCM, minichromosome maintenance, PP, phosphatase.
Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U46131).

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coordinated with the periodic activation of cyclin-dependent kinases. Screens in the yeast *Schizosaccharomyces pombe* to isolate mutants that undergo multiple rounds of replication in the absence of intervening mitosis have identified unambiguously the mitotic kinase cdc2/cyclinB as an inhibitor of illegitimate initiation of S phase. In this screen several alleles of cdc2 and one allele of cdc13, the *Sch. pombe* homolog of cyclinB, were isolated (17, 18). Most convincing is the evidence that deletion of cdc13 (which is required solely at the G_2/M transition in *Sch. pombe*) will lead to a dramatic increase in the ploidy of the mutant (18). Moreover, mutations in the rum1 gene, a cdc2/cyclinB inhibitor, allow multiple rounds of DNA synthesis within one cell cycle (19).

Here, we describe the behavior of MCM4, a new member of the MCM family from *Xenopus*. MCM4 is a component of the large MCM complex required for DNA replication, which is phosphorylated in a cell cycle-dependent fashion by cdc2/cyclinB kinase. This phosphorylation is very specific for this cdk and decreases the ability of the whole MCM complex to bind to chromatin, providing a biochemical explanation for the inactivation of the MCM complex and for its clearing from the chromatin as replication proceeds during S phase. Our findings provide an explanation for the mechanism of action of the MCM complex and for the resetting of its activity at each cell cycle, in addition to an interpretation of the re-replication phenotype observed in *Sch. pombe* cdc2 and cdc13 mutants.

MATERIALS AND METHODS

Cloning and Sequencing of *Xenopus laevis* MCM4. A λGT10 cDNA library from stage 17 *Xenopus* embryos was screened with two 60-mer oligonucleotides taken from a *Xenopus* partial cDNA sequence (10) (EMBL accession no. Z15033). The oligonucleotides used to probe the library are nonoverlapping sequence from a region of strong homology among previously identified members of the MCM family of proteins. One million plaques were screened and produced 110 clones that hybridized strongly with both probes after two rounds of plaque purification. Clone no. 26, with a 3.5-kb insert, was subcloned into the *Eco*RI site of the Bluescript KS+ vector (Stratagene) and sequenced using an Applied Biosystem Automated Sequencer. The full-length open reading frame (2.5 kb) of this clone was found to be the *Xenopus* MCM4 homolog by sequence comparison with other related family.

Protein Sample Preparation from Fertilized Eggs. Freshly squeezed *Xenopus* eggs were synchronously fertilized with perforated testes. Ten eggs per sample were collected at 10-min intervals and snap frozen in liquid nitrogen. The eggs were crushed in 100 μl of EB (0.5% Nonidet P-40/20 mM Tris, pH 7.4/100 mM NaCl/5 mM EDTA/1 μg/ml protease inhibitors), spun at 10,000 × *g* at 4°C for 10 min in Eppendorf tubes to separate yolk proteins and lipid and the supernatant was collected. Protein extract (10 μl) from each sample was electrophoresed on an 8% polyacrylamide gel, transferred to nitrocellulose, probed with either the MCM4 or cyclin B2 antibody (20), and visualized as described below.

***Xenopus* Cell Cycle Extracts.** Interphase, metaphase-arrested, and cycling extracts were made according to methods described (21). Nuclei were isolated from cycling extracts (14) with some modification. Samples were centrifuged through 400 μl nuclear isolation buffer containing 0.1% Nonidet P-40 and 30% sucrose. Duplicate samples were incubated for an additional 40 min at 30°C with cdc2/cyclinB kinase before centrifugation. All samples were resuspended in sample buffer, electrophoresed on an 8% polyacrylamide gel, transferred to nitrocellulose, probed with the MCM4 antibody and visualized as described below.

Antibody Production. Keyhole limpet hemocyanin-coupled antigenic peptide sequence PRGQYTSGKGSGAV (amino acids 527–541) (synthesized by E. Heimer at Hoffmann–LaRoche Research Division) was used to immunize two rabbits for the production of polyclonal antibodies. Immunization and monthly boosts were performed as per standard protocol for Pocono Rabbit Farm (Canadensis, PA). Supernatant from the caprylic acid precipitation of crude serum was ammonium sulfate precipitated according to standard procedures, resuspended in PBS, and then passed over a peptide coupled CNBr Sepharose column to affinity purify the antibody. Two rabbits gave strong immune response to peptide and each were purified by this protocol.

MCM3 Immunoprecipitations and Gradient Gel Immunoblots. Aliquots (100 μl) of *Xenopus* cell cycle extract were incubated with 20 μl of affinity-purified MCM3 antibody (13) for 4 hr followed by 1 hr incubation with 100 μl protein A Sepharose beads, both at 4°C with rotation. Immune complexes were collected by brief centrifugation in Eppendorf tubes at 10,000 × *g* and washed three times in buffer containing 150 mM NaCl/50 mM Tris·HCl, pH 8.0/0.5% Nonidet P-40 then resuspended in sample buffer. Samples were electrophoresed on an SDS/8% polyacrylamide gel and transferred to nitrocellulose membrane by semi-dry transfer method. Immunoblot was probed with MCM antibodies, enhanced with horseradish peroxidase-protein A and visualized using enhanced chemiluminescence (Amersham).

For native gradient gel electrophoresis samples were collected at 20-min time intervals from an activated *Xenopus* cell cycle extract (21), resuspended in 1× TBE containing 10% glycerol, and electrophoresed on a 4–29% nondenaturing linear gradient polyacrylamide gel in 0.5× TBE, run overnight in 1× TBE. Prior to transfer the gel was soaked in SDS buffer (48 mM Tris/39 mM glycine/0.25% SDS) for 30 min with agitation followed by a 30-min soak at 80°C. Proteins were transferred to nitrocellulose membrane by semi-dry method as previously described, probed with the MCM4 antibody enhanced with horseradish peroxidase-anti-rabbit antibody and visualized using enhanced chemiluminescence.

Histone H1 Kinase and DNA Replication Assays. Total H1 kinase activity was measured according to standard protocols (21). Cdc2 H1 kinase activity is determined by immunoprecipitating cdc2/cyclinB from extract using a specific *Xenopus* cdc2 antibody (22) then following the standard protocol for histone H1 kinase assay using the immunoprecipitates.

To monitor DNA replication in cycling extracts 4 μl aliquots were taken at intervals and frozen in liquid nitrogen. Samples were thawed on ice, supplemented with 1 μl of [^{32}P]dATP diluted 1:10 in extract buffer (21), then incubated at 22°C for 80 min. Replication was stopped by addition of 100 μl stop buffer (20 mM Tris·HCl/5 mM EDTA, pH 7.5/0.5% SDS). All samples were then mixed with 4 ml of cold 10% trichloroacetic acid to precipitate the nucleic acids then processed using a vacuum manifold.

Cyclin-Dependent Kinase Purification. cdc2, cdk2, cdk4, cyclinB, cyclinA, cyclinE, and cyclinD1 cloned in baculovirus vector (23) were a generous gift of Wade Harper (Baylor University, Houston). They were expressed by pairs in insect cells: cdc2/cyclinB; cdk2/cyclinA; cdk2/cyclinE, and cdk4/cyclinD1. The complexes were purified on gluthation agarose (23).

***In Vitro* Phosphorylation of ^{35}S-Labeled MCM4.** MCM4 cDNA was subcloned into a T7 vector. The fragment was inserted into *Nco*I–*Xho*I sites.

Synthetic mRNAs were made by *in vitro* transcription with T7 polymerase using a kit from Ambion, following the manufacturer's instructions. [^{35}S]Methionine-labeled MCM4 protein was generated by *in vitro* transcription in rabbit reticulocyte lysate (Promega). In a typical experiment, 30 μl of ^{35}S-labeled MCM4 were immunoprecipitated with 15 μl of affinity purified MCM4 antibodies for 1 hr at 4°C. The immunocomplexes were then immobilized on protein A-Sepharose beads (Pharmacia), washed three times, aliquoted, and processed. Aliquots equivalents to 3–4 μl of translation products were typically incubated in 20 μl containing 2 μl of the diluted kinase, 100 mM ATP in kinase buffer (23). The reactions were carried out at 30°C for 15 min. The phosphorylated MCM4 could then be further treated with phosphatase (PP) 2A (Upstate Biotechnology, Lake Placid, NY)

in combination or not with Inhibitor2 (Upstate Biotechnology) or 5 μM okadaic acid. The reactions were stopped by addition of PAGE sample buffer and processed for electrophoresis.

[^{32}P]ATP Phosphorylation of MCM4. *In Vitro.* The MCM complex was first immunoprecipitated with affinity purified MCM3 antibodies (14), the complex was either directly phosphorylated by the different cdks, in similar conditions as described above in presence of 10 mCi (1 Ci = 37 GBq) of [^{32}P]ATP. Alternatively, the MCM complex was dissociated in SDS, diluted 100-fold and MCM4 was further immunoprecipitated using affinity purified MCM4 antibodies. This was then subjected to phosphorylation as described above. These two methods gave identical results. We were not able to immunoprecipitate the MCM complex directly using anti-MCM4 antibodies, whilst the same antibodies could immunoprecipitate quantitatively the translated MCM4 product. We conclude that the epitope recognized by the MCM4 antibodies is not accessible in the native complex.

In Vivo. Metaphase-arrested extracts (22) were labeled for 1–2 hr depending on the experiments with [^{32}P]orthophosphate. In a typical experiment, 200 μl of extract were incubated with 5 mCi of ^{32}P. ^{32}P-labeled MCM4 was then immunoprecipitated from the extract as described above.

Two-Dimensional Peptide Mapping of Phosphorylated MCM4. The *in vitro* and *in vivo* labeled products were prepared as described. The labeled MCM4 was run on a 8% polyacrylamide gel and transferred onto nitrocellulose membranes. The phosphopedtide analysis, was done following trypsin digestion, using a HTLE-7000 apparatus (C.B.S. Scientific), following the manufacturer's instructions.

RESULTS

Identification of *Xenopus* MCM4, a Member of the MCM Family. Using a previously published partial sequence of a *Xenopus* MCM homolog (10) we isolated a novel *Xenopus* MCM cDNA with a putative open reading frame of 863 amino acids. This *Xenopus* MCM is most related to the cdc21 gene from *Sch. pombe* (10) and the CDC54 gene from *Sac. cerevisiae* (9). From the comparison between our clone and the other known members of the MCM family we concluded that clone 26 encoded a *Xenopus* homolog of Spcdc21/ScCDC54, which we named MCM4. This clone is more than 99% identical with a *Xenopus* cDNA [GenBank U29178 (27)], differs only at amino acid residues 73, 739, and 740. Like all MCM family members, *Xenopus* MCM4 shows a high degree of identity with the other members in the core region "MCM box," with similarity outside of this region seen only with Spcdc21 and ScCDC54. Interestingly, *Xenopus* MCM4 possesses a cluster of putative cdc2 phosphorylation sites in the N-terminal part of the protein. Although, the exact position of these sites is not conserved, all members of the MCM4 subfamily (human, mouse, fission yeast, and budding yeast) show a group of sites within the first 150 amino acids of the protein. This N-terminal cluster of cdc2 phosphorylation sites is not found in the other MCM family members.

Association of MCM4 with Other Known MCMs. We generated several batches of rabbit polyclonal antibodies against the *Xenopus* MCM4 protein (see *Materials and Methods*). In this study, unless otherwise mentioned, we used a polyclonal antibody that recognizes specifically the *Xenopus* MCM4 protein. Another polyclonal antibody was also made that does not display specificity for a particular MCM protein and recognizes at least four members of the MCM family (see *Materials and Methods* and Fig. 1*A*, lane 1).

Using a specific antibody against *Xenopus* MCM3 (14) to immunoprecipitate MCM3 and MCM3-associated proteins from *Xenopus* extracts, followed by Western blotting with specific antibodies against the MCM4 protein, we demonstrate that MCM4 is associated with MCM3 (Fig. 1*A*, lane 3). Furthermore, using an antibody directed against the *Drosophila* homolog of MCM5 (11) we show that this MCM3/MCM4 complex also contains the *Xenopus* homolog of MCM5 (Fig. 1*A*, lane 4). Our second polyclonal antibody recognizes all three identified *Xenopus* MCMs (MCM3, MCM4, and MCM5) as well as an additional yet unidentified member of the MCM family (Fig. 1*A*, lane 1). Therefore, we have identified MCM4 as another subunit of the MCM complex that has not been characterized previously.

Using nondenaturing gel electrophoresis followed by Western blot analysis (see *Materials and Methods*) MCM-containing complexes can be resolved as two protein complexes of ≈500–600 kDa, respectively (Fig. 1*B*), with the 600-kDa complex being the most abundant. The molecular mass of each MCM member is about 100 kDa, and if these complexes are exclusively composed of MCM proteins, they should contain five and six subunits, respectively, of which we have identified three. This further demonstrates that MCMs assemble into high molecular mass complexes in *Xenopus*.

One of the original postulates about the replication licensing factor is that it needs to be inactivated, destroyed, or disassembled at each cell cycle. We tested whether the abundance of the complexes changed during the cell cycle by following their profiles throughout the cell cycle in *Xenopus* metaphase-arrested extracts activated by Ca^{2+}. We did not observe any noticeable difference in the amount of each high molecular mass complex or in the ratio between complexes throughout the cell cycle (Fig. 1*B*). This observation eliminates the possibility that the MCM protein complexes are disassembled or destroyed (even in part) during the cell cycle. Therefore, the inactivation of the MCM complex is achieved through other mechanisms.

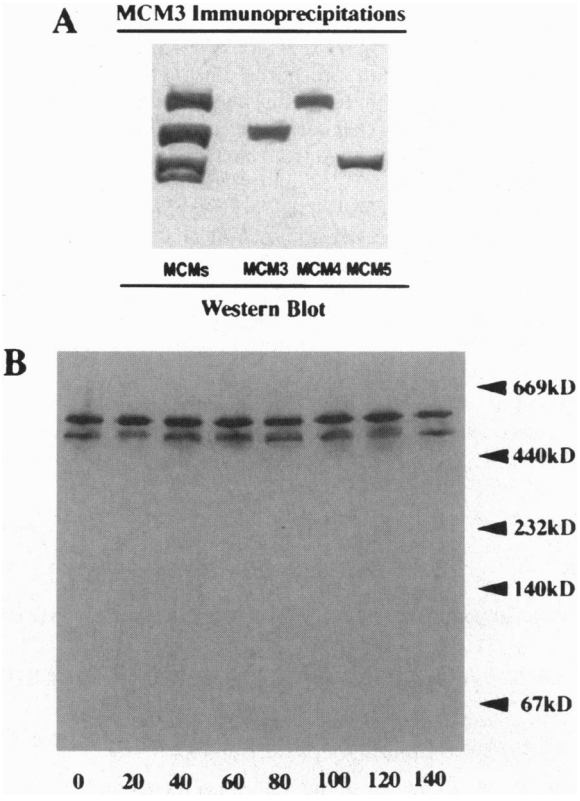

FIG. 1. MCM4 and the MCM complexes. (*A*) Immunoprecipitation of the MCM complex with MCM3 antibody was followed by SDS/PAGE and Western blot analysis using different MCM antibodies: lane 1, anti-MCM antibody with no subfamily specificity; lane 2, MCM3 antibody; lane 3, MCM4 antibody; Lane 4, MCM5 antibody. (*B*) Resolution of two discrete MCM4-containing complexes by native acrylamide gradient gel. Samples are metaphase-arrested extract (0 time point) and time points following activation by calcium taken at 20-min intervals.

Cell Cycle-Dependent Regulation of MCM4. We asked whether the inactivation of the MCM complexes at each cell cycle could be achieved by posttranslational modifications of one or several of its subunits.

When we followed the behavior of the MCM4 monomer by PAGE, we observed a cell cycle-dependent change in mobility of the protein (Fig. 2*A* and *B*). Fig. 2*A Upper*, shows the changes in mobility of MCM4 observed during the cell cycle following fertilization. As unfertilized eggs, arrested in metaphase of meiosis II, enter interphase of the first cell cycle, MCM4 shifts from a slow migrating to a fast migrating form. When the eggs proceed through S phase and enter the next M phase (at 50–60 min), MCM4 shifts back to the slower form. Similar changes in mobility of MCM4 can be demonstrated in activated "cycling extracts" (Fig. 2*B Top*). In this case, we followed MCM4 behavior through two complete cell cycles. The MCM4 shift to a slower form starts in mid-S phase and is complete at the time of M phase, when all the MCM4 protein is shifted. It is important to note that the shift in mobility of MCM4 starts during S phase when the total level of histone H1 kinase (Fig. 2*B Middle*) and cdc2 kinase (Fig. 2*B Bottom*) are still low compared with the M phase levels. We observed a similar MCM4 mobility shift in oocytes induce to mature, *in vitro*, by progesterone and progress from G_2 to M phase (data not shown).

Identification of the Protein Kinase Responsible for MCM4 Phosphorylation. The cell cycle-dependent changes in the electrophoretic mobility of MCM4 prompted us to investigate the possible role of members of the cyclin-dependent protein kinase family in phosphorylating MCM4.

In a first series of experiments, we subcloned *Xenopus* MCM4 cDNA in a vector allowing high yield *in vitro* transcription (see *Materials and Methods*). This synthetic RNA was used to produce ^{35}S-labeled MCM4 in rabbit reticulocyte lysate. Labeled MCM4 product was then immunoprecipitated using the MCM4 specific antibody and subjected to different combinations of protein kinases and protein phosphatases (Fig. 3*A*). The enzymes used in this study were highly purified from baculovirus infected cells (see *Materials and Methods*). Cdc2/glutathione *S*-transferase (GST)-cyclinB, cdk2/GST-cyclinA, cdk2/GST-cyclinE, and GST-cdk4/cyclinD1 were produced by coinfection of sf9 cells as described (23). Phosphorylation of MCM4 by the cdc2/cyclinB complex but not by either, cdk2/cyclinA, cdk2/cyclinE, or cdk4/cyclinD1 complexes promoted a shift in mobility of the protein (Fig. 3*A*, lanes 3–6). The amounts of protein kinases used in this experiment were normalized using histone H1 as a substrate for cdc2/cyclinB, cdk2/cyclinA, and cdk2/cyclinE and using the truncated form of the retinoblastoma protein p56Rb as a substrate for cdk4/cyclinD1. This change in mobility is due to phosphorylation as seen by the effect of PP2A and of a combination of PP2A with specific inhibitors on the phosphorylated MCM4 (Fig. 3*A*, lanes 7–9).

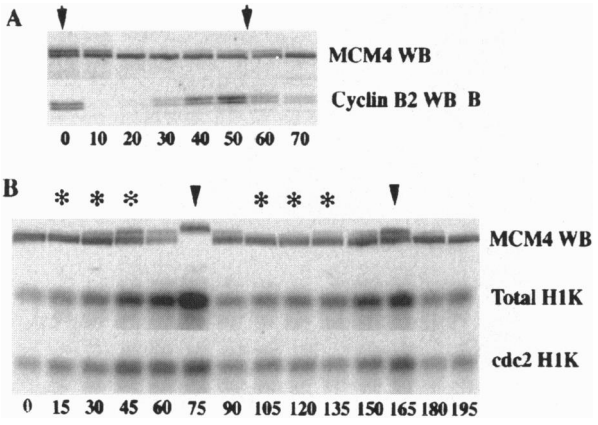

FIG. 2. Cell cycle regulation of MCM4. (*A*) Pools of synchronized fertilized eggs were lysed at 10-min intervals and subjected to Western blot analysis with MCM4 antibody (*Upper*) or cyclinB antibody to assess the stage of the cell cycle (*Lower*). The arrowheads indicate M phase. (*B*) Aliquots from cycling extracts taken at 15-min intervals were subjected to Western Blotting with MCM4 antibody (*Top*), measurement of histone H1 kinase activity (*Middle*) and immunoprecipitation by a C-terminal peptide antibody, specific for cdc2 followed by histone H1 kinase assays (*Bottom*). Arrowheads indicate M phase, the asterisks indicate S phase.

We demonstrated more directly that cdc2/cyclinB protein kinase can phosphorylate MCM4 *in vitro* by monitoring the incorporation of ^{32}P. Again, cdc2/cyclinB and not cdk2/cyclinA, cdk2/cyclinE, or cdk4/cyclinD1 was able to phosphorylate MCM4 *in vitro* (data not shown). MCM4 was the only MCM phosphorylated by cdc2 kinase in the complexes immunoprecipitated by MCM3. The ^{32}P-labeled band following cdc2 phosphorylation (Fig. 3*B*, lane 1) corresponds to MCM4 as assessed by Western blot analysis of the MCM3 immunoprecipitates with MCM3 (lanes 6 and 7), MCM4 (lanes 4 and 5), MCM5 (lanes 8 and 9) specific antibodies, and the pan-MCMs (lanes 2 and 3) antibody. In addition MCM4 is the only MCM displaying a shift in mobility following phosphorylation by cdc2 (compare lanes 3 and 5 with lanes 2 and 4).

We asked whether the *in vitro* phosphorylation of MCM4 was relevant to the *in vivo* phosphorylation observed during the cell cycle in eggs or in cell-free extracts. We compared the two-dimensional phosphopeptide maps following tryptic digestion of MCM4 phosphorylated *in vitro* by cdc2/cyclinB kinase or *in vivo* in metaphase-arrested extracts (see *Materials and Methods*). As seen in Fig. 3*C*, the two-dimensional tryptic maps are almost identical and superimposable, with the exception of one single phosphopeptide that exhibited a different mobility *in vivo* and *in vitro* (marked with arrows on Fig. 3*C*). This peptide displays an increased mobility following *in vitro* phosphorylation that is due to an additional phosphorylation on the same peptide taking place *in vitro* but not *in vivo*. It is quite common to observe that a protein kinase phosphorylates one or several additional amino acid on a given peptide. We assume that this additional *in vitro* phosphorylation is not physiologically relevant.

This clearly demonstrates that MCM4 is phosphorylated *in vivo* at the same sites as it is phosphorylated *in vitro* by cdc2/cyclinB and therefore that cdc2 is the kinase responsible for the cell cycle phosphorylation of MCM4. In addition, cdc2/cyclinB is the only cdk that can promote the shift in mobility of MCM4.

Phosphorylation of MCM4 Reduces Its Affinity for Chromatin. In yeast, MCM genes are all required for the entry into S phase, in addition, depletion of the MCM complex from *Xenopus* extracts prevent the replication of added G_2 nuclei (13). These observations demonstrate that MCM proteins are required for initiation of DNA replication in *Xenopus* and prompted us to investigate the potential association of MCM4 with chromatin. We followed the ability of the MCM4 protein to associate with chromatin throughout the cell cycle. In these experiments, cycling extracts were supplemented with *Xenopus* sperm nuclei (see *Materials and Methods*). We followed the association of MCM4 with the chromatin by purifying the assembled nuclei away from the extract and blotting them with the MCM4-specific antibody (Fig. 4 *Middle*). Although, the total levels of MCM4 do not change in the extract throughout this period (Fig. 1*B*), MCM4 is found associated with chromatin only during early to mid-S phase. Similar observations were made for MCM3 (13, 14). The clearing of MCM4 from the chromatin coincides exactly with the change in mobility of MCM4 protein on PAGE at mid-S phase (Fig. 2*B*). This strongly suggests that MCM4 phosphorylation is responsible for the disassembly of MCM4 from the chromatin. In an attempt to demonstrate the biological relevance of MCM4 phosphorylation by cdc2/cyclinB, we investigated the effect of this phosphorylation on the affinity of MCM4 for the chromatin.

At each time point the purified nuclei were subjected to either kinase buffer alone (Fig. 4 *Middle*) or to cdc2/cyclinB phosphorylation (Fig. 4 *Lower*). The treatment of MCM

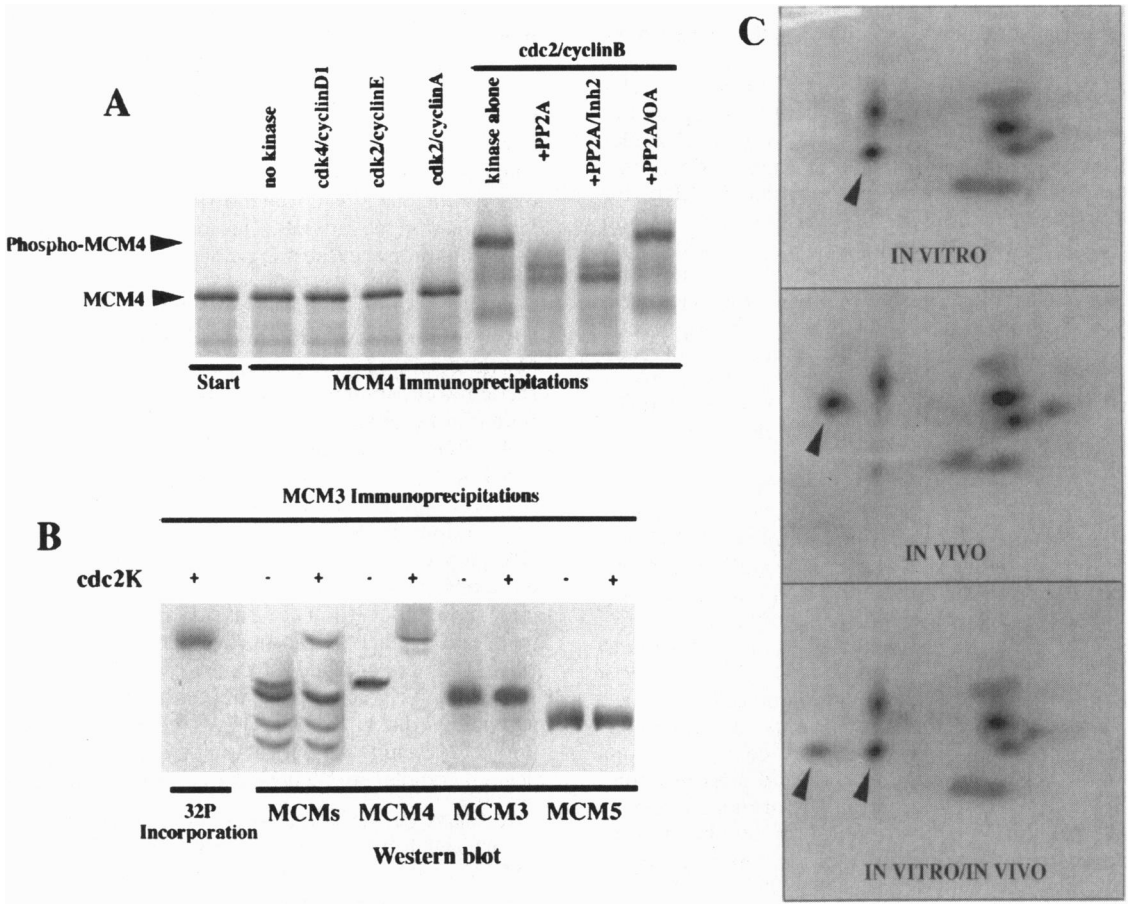

FIG. 3. Phosphorylation of MCM4 by cdc2/cyclinB. (*A*) *In vitro* phosphorylation of the [^{35}S]methionine-labeled MCM4. Lane 1 is the starting material, the *in vitro* translation of MCM4 synthetic mRNA. The following lanes 2–9 are MCM4 immunoprecipitations subjected to various treatments, as indicated. The amounts of protein kinases used were normalized for their activity. All enzymatic treatments were done on MCM4 immobilized on beads. Lanes 6–9 have all been treated with cdc2/cyclinB first, then with PP2A (lane 7), PP2A and inhibitor 2, a phosphatase 1 inhibitor (PP2A plus inh2, lane 8), PP2A and okadaic acid, a phosphatase 2A inhibitor (PP2A plus OA, lane 9). (*B*) MCM complexes immunoprecipitated using MCM3 antibodies were subjected to phosphorylation with [γ-^{32}P]ATP by cdc2 protein kinase (lane 1). Similar complexes were subjected to Western blot analysis, following treatment (+) or no treatment (−) with cdc2 in the presence of cold ATP. Lanes 2 and 3 were blotted with the antibody that recognize the MCM family of proteins. Lanes 4 and 5 were blotted with MCM4 antibody. Lanes 6 and 7 were blotted with MCM3 antibody. Lanes 8 and 9 were blotted with MCM5 antibody. (*C*) Two-dimensional phosphopeptide analysis of ^{32}P-labeled MCM4 following tryptic digest. *In vitro* phosphorylation (*Top*), *in vivo* phosphorylation (*Middle*), and *in vitro* and *in vivo* phosphorylation comigrations (*Bottom*). Chromatography was performed along the vertical axis, electrophoretic separation along the horizontal axis. The arrows indicate the phosphopeptide displaying altered mobility when phosphorylated *in vitro*.

complexes associated with chromatin by cdc2/cyclinB abolished the association of MCM4 to the chromatin, which was reduced to a non specific background binding. This phosphorylation also abolished the binding of the MCM protein complex from the chromatin, as assessed by MCM3 Western blot (data not shown) thus confirming that MCM4 phosphorylation leads to clearing of the entire MCM complex from chromatin rather than MCM4 alone.

DISCUSSION

We have identified and characterized MCM4, a member of the MCM family most related to Spcdc21/ScCDC54. We show that MCM4 is part of at least two protein complexes comprising MCM3, MCM5, and other yet unidentified components (Fig. 1*A*). We calculated that the M_r of these complexes is 500 and 600 kDa, respectively; they could therefore contain five or six MCM subunits of ≈100 kDa each (the average molecular weight of MCM proteins). The work of others (12, 14) has shown that the MCM3 containing complex, the same as we describe here, is necessary for initiation of DNA replication and possesses some replication licensing factor characteristics. One possible way of regulating the activity of such a factor could be by cell cycle regulated destruction, as in the case of cyclins, or cell cycle regulated assembly and disassembly of the whole complex. We show here that this is not the case, since the complex is stable

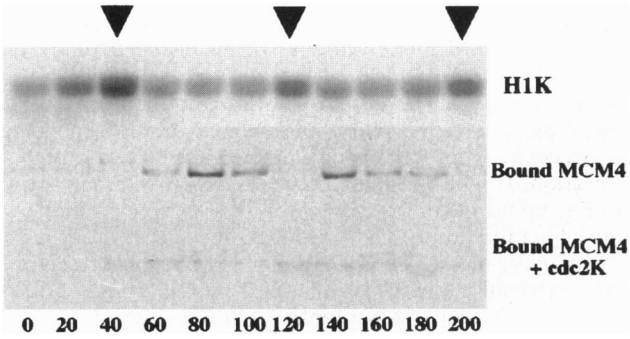

FIG. 4. Inhibition of MCM4/chromatin association by cdc2/cyclinB. Aliquots of cycling extracts supplemented with nuclei were taken at 20-min intervals. The total histone H1 kinase in the cycling extract is shown (*Top*). The extract was incubated with sperm nuclei, the nuclei were purified at 20-min intervals, then treated with kinase buffer alone, purified again, and blotted with MCM4 antibody (*Middle*), or treated with cdc2/cyclinB in kinase buffer, repurified, and blotted with MCM4 antibody (*Bottom*).

throughout the cell cycle of *Xenopus*. In the absence of complex disassembly or destruction, we favor the possibility of posttranslational modification of one or several of its subunits.

We show that MCM4 is phosphorylated in a cell cycle-dependent manner starting in S phase and peaking at the G_2/M transition of the cell cycle. We observe this cell cycle-dependent phosphorylation in various experimental situations: during oocyte maturation—i.e., the meiotic cell cycle (data not shown), during the early mitotic cell cycle of the embryo, and finally in cycling cell-free extracts made from synchronized activated *Xenopus* eggs. A similar observation of posttranslational modifications during the *in vivo* early mitotic cell cycle was recently reported (24). The change in electrophoretic mobility observed following *in vitro* treatment with cdc2 protein kinase is due to phosphorylation as seen by the effect of PP2A, a very specific protein phosphatase (Fig. 3*A*, lane7). Although, this phosphorylation starts before the bulk of cdc2 protein kinase is activated, we found compelling evidence for the direct phosphorylation of MCM4 by cdc2 protein kinase *in vivo*. Firstly, cdc2 protein kinase is the only cyclin-dependent kinase able to promote the shift in mobility of MCM4, excluding the possibility that MCM4 phosphorylation might be due to another cdk active during that part of the cell cycle such as cdk2/cyclinA or cdk2/cyclinE. Second, cdc2/cyclin B phosphorylates MCM4 *in vitro* at identical sites to those phosphorylated *in vivo*.

In addition, the analysis of the primary amino acid sequence of MCM4 shows several canonical consensus sites for phosphorylation by cdc2, some of these sites are conserved from yeast to *Xenopus*. The fact that the clustered sites are conserved in other species strongly argues in favor of a conserved mechanism for inhibiting DNA replication by the mitotic kinase from mid-S phase through G_2 in different organisms. This cluster of cdc2 phosphorylation sites is not found in the other MCMs.

We have therefore identified a new substrate for cdc2/cyclinB. The demonstration that cdc2 phosphorylates MCM4 is a critical finding that links the cell cycle engine with the DNA replication machinery. MCM4 is a rather unusual substrate since it is very specific for cdc2/cyclinB even *in vitro*. We calculated that cdc2/cyclinB phosphorylates MCM4 at least 50-fold more efficiently than cdk2/cyclinA and at least 200-fold more efficiently than cdk2/cyclinE.

Although, we cannot exclude the possibility that another cyclin dependent kinase can phosphorylate MCM4, we believe that such a phosphorylation, if it exists should be of minor amplitude. We demonstrate that there is no prerequisite phosphorylation of MCM4 by other protein kinases to render it a substrate for cdc2/cyclinB, since *in vitro* translated MCM4 can be phosphorylated *in vitro* as a monomer by highly purified cdc2/cyclinB kinase. Experiments in *Xenopus* extracts (25, 26) have shown that depletion of cdks (probably cdc2 and cdk2) will prevent DNA replication in extracts, suggesting that a positive role for cdk phosphorylation in initiation of DNA replication. Therefore, phosphorylation by cyclin-dependent kinases could exert both positive and negative effect on DNA replication. It will be important to establish whether MCM proteins could be the target(s) for the phosphorylation required for initiation of DNA replication.

We propose that the phosphorylation of MCM4 by cdc2 is one of the mechanisms by which the cdc2 protein kinase inhibits illegitimate initiation of DNA replication during late S phase and G_2. The phosphorylation of MCM4, as seen by the shift in mobility, starts exactly at the time of MCM4 is cleared from the chromatin. Moreover, phosphorylation of the MCM complex associated with chromatin corresponds to its clearing from the chromatin. In addition, MCM4 is the component of the MCM complex that is phosphorylated by cdc2 (Fig. 3*B*). These facts strongly argue that the function of cdc2 phosphorylation of MCM4 is to prevent multiple rounds of replication by preventing the binding of MCM4 during late S phase and G_2. One implication of this observation is that low levels of cdc2 kinase in S phase and G_2 are not physiologically irrelevant, as it is normally thought. This level of kinase activity, although unable to trigger the G_2/M transition, could mediate inhibition of re-replication. It is also important to point out that the phosphorylation of MCM4 changes its affinity for the chromatin but does not promote the dissociation of MCM4 from the MCM complex or its dissociation into subunits (see Fig. 1*B*). This implies that the phosphorylation of MCM4 promotes the release of the whole MCM complex from the chromatin.

However, it is possible that MCM4 is not the only target of cdc2 for preventing reinitiation. Other proteins that assemble at the origin of replication such as subunits of the origin recognition complex or the homolog of cdc18/CDC6 are also potential targets.

Additional arguments in favor of this hypothesis come from experiments in other systems. In *Sch. pombe*, it has been proposed that the low levels of cdc2/cdc13 (the equivalent of cdc2/cyclinB) observed in late S phase and G_2 are responsible for inhibiting DNA replication. Here, we provide a biochemical explanation for these observations (17, 18). We believe that the "re-replication" phenotype observed in cdc2 mutants or in cdc13 deletion could be due to the lack of phosphorylation of cdc21 (the *Sch. pombe* MCM4) by the cdc2/cdc13 kinase.

The specificity of the phosphorylation of MCM4 by cdc2 kinase and not by cdk2 kinase is also in agreement with the observation that cdc2/cyclinB kinase will inhibit the assembly of the prereplication complex in *Xenopus* extract while cdk2 will not (27).

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