Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 14693–14698, December 1998
Biochemistry

Protein phosphatase 2A is required for the initiation of chromosomal DNA replication

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Communicated by Tony Hunter, The Salk Institute for Biological Studies, San Diego, CA, October 13, 1998 (received for review August 20, 1998)

ABSTRACT **Protein phosphatase 2A (PP2A) is an abundant, multifunctional serine/threonine-specific phosphatase that stimulates simian virus 40 DNA replication. The question as to whether chromosomal DNA replication also depends on PP2A was addressed by using a cell-free replication system derived from *Xenopus laevis* eggs. Immunodepletion of PP2A from *Xenopus* egg extract resulted in strong inhibition of DNA replication. PP2A was required for the initiation of replication but not for the elongation of previously engaged replication forks. Therefore, the initiation of chromosomal DNA replication depends not only on phosphorylation by protein kinases but also on dephosphorylation by PP2A.**

The initiation of chromosomal DNA replication in eukaryotes can be divided into two general steps (reviewed in refs. 1 and 2). The first occurs during the G_1 phase of the cell cycle and involves the formation at the origin of a prereplication complex (pre-RC) containing the origin recognition complex (ORC), cdc6, and a complex of minichromosome maintenance proteins (MCM). The second occurs during the G_1/S transition, when cyclin-dependent kinases as well as the cdc7/dbf4 kinase convert the pre-RC into an active replication fork by a currently unknown mechanism. There is evidence that the substrate of cdc7/dbf4 may be the MCM complex (3, 4), whereas the cyclin-dependent kinase substrates are not known.

Whether cellular DNA replication also might be regulated by protein phosphatases is not known. However, *in vitro* studies on the replication of simian virus 40 (SV40) DNA, which depends on various cellular DNA replication factors as well as SV40 large T antigen, have shown that the catalytic C subunit of PP2A stimulates SV40 DNA replication (5, 6). Protein phosphatase 2A (PP2A) dephosphorylates SV40 large T at serines 120, 123, 677, and 679 (7, 8). Dephosphorylation of these residues facilitates the interaction of two large T hexamers at the replication origin, leading to the unwinding of origin DNA (9). Interestingly, whereas phosphorylation at serine residues inhibits the large T replication function (10–12), phosphorylation at Thr-124 is absolutely required for it (11, 13). Thus, SV40 large T is regulated positively and negatively by protein kinases in addition to being stimulated by PP2A.

PP2A consists of three subunits, a 36-kDa catalytic C subunit and a 65-kDa regulatory A subunit, which form the core enzyme, and a regulatory B subunit that binds to the core enzyme yielding the holoenzyme (reviewed in ref. 14). Both the A and C subunits exist as two isoforms (α and β), whereas the B subunits fall into three families designated B, B′ (also called B56), and B′′, which are unrelated by protein sequence (15–24). The combination of these different subunits could give rise to many forms of PP2A, which may differ in substrate specificity (14, 25), subcellular localization (20, 22), or tissue-specific expression (19–22). A fourth class of proteins able to associate with PP2A core enzyme is the tumor antigens encoded by polyomaviruses; these antigens are unrelated by sequence to the three families of B subunits (reviewed in ref. 26).

To investigate whether PP2A is involved in the replication of chromosomal DNA, we used the *Xenopus* cell-free replication system (27, 28). We found that removal of PP2A from egg extract caused inhibition of DNA replication and that PP2A was required for initiation, but not elongation, of DNA replication.

MATERIALS AND METHODS

Preparation of Egg Extract. *Xenopus laevis* frogs were purchased from Xenopus I (Ann Arbor, MI). Unfractionated egg extracts, fractionated membrane-free cytosol, and purified membranes were prepared as described (29). Briefly, frogs were primed by injecting each frog with 100 units of pregnant mare serum gonadotropin (Calbiochem) 2–7 days before eggs were required. To induce ovulation, each frog was injected with 400 units of human chorionic gonadotropin 20 h before collecting eggs. Jelly was removed from eggs in 2% cysteine and then washed three times in one-quarter strength modified amphibian Ringer's solution (100 mM NaCl/2 mM KCl/1 mM $MgSO_4$/2 mM $CaCl_2$/0.1 mM EDTA/5 mM Hepes, pH 7.8) and egg-lysis buffer (250 mM sucrose/2.5 mM $MgCl_2$/50 mM KCl/10 mM Hepes, pH 7.7/50 μg/ml cycloheximide/1 mM DTT). Eggs were packed by centrifugation at 700 $\times$ *g* in a Falcon tube. The excess buffer was removed, and leupeptin, aprotinin, and cytochalasin B were added to final concentrations of 5.0, 5.0, and 2.5 μg/ml packed eggs, respectively. The eggs were crushed by centrifugation at 16,000 $\times$ *g* at 4°C for 15 min in a JS-13 rotor in a Beckman J2-21 centrifuge. The crude cytoplasmic layer (unfractionated egg extract) was withdrawn with a 21-gauge needle and supplemented with leupeptin, aprotinin, cytochalasin B, cycloheximide, and nocodazole to final concentrations of 10, 10, 5, 50 and 3.3 μg/ml, respectively. DTT also was added to 1 mM. Egg cytosol and purified membranes were prepared from unfractionated egg extract by centrifugation at 55,000 rpm at 2°C for 90 min in a TL-55 swinging bucket rotor with a Beckman TL-100 ultracentrifuge.

The preparation of nucleoplasmic extract (NPE) has been described (29). Briefly, unfractionated egg extract was supplemented with nocodazole to a concentration of 3.3 μg/ml and centrifuged at 16,000 $\times$ *g* at 4°C for 10 min. The recovered extract was supplemented with an ATP-regenerating system

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Abbreviations: MCM, a complex of minichromosome maintenance proteins; NPE, nucleoplasmic extract; ORC, origin recognition complex; PP2A, protein phosphatase 2A; pre-RC, prereplication complex; SV40, simian virus 40.

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and sperm from which membranes had been removed at a concentration of 4,000 sperm per μl. The mixture was incubated at room temperature for 80 min and centrifuged at 16,000 $\times$ *g* at 4°C for 2 min. The nuclear layer at the top of the tube was collected and centrifuged at 55,000 rpm at 2°C for 30 min in a TL-100 centrifuge. After centrifugation, the lipid at the top of the sample was removed, and the clear NPE layer was harvested.

Immunodepletion and Western Blotting. PP2A was depleted from extracts by using rat mAb 6F9 directed against the N terminus of the human Aα subunit, coupled to protein G Sepharose as described (30). mAb 6F9 precipitates PP2A core enzyme and holoenzyme. More than 95% of PP2A was removed by three consecutive immunodepletions of 1 h each at 4°C. Typically, 100 μl of egg cytosol was supplemented with nocodazole (final concentration was 3.3 μg/ml) and mixed with 35 μl of mAb 6F9-coupled Sepharose beads for 1 h at 4°C. The mixture was centrifuged at 14,000 rpm for 3 min in a Eppendorf 5402 centrifuge. The recovered supernatant was mixed with 30 μl of mAb 6F9-coupled Sepharose beads for 1 h at 4°C. After centrifugation, the recovered supernatant was mixed with 25 μl of mAb 6F9-coupled Sepharose beads for 1 h and centrifuged to recover the supernatant. Mock-depleted extract was prepared the same way by using rat IgG-coupled protein G Sepharose. To monitor the extent of depletion, serial dilutions of undepleted extract were analyzed in parallel with the depleted extracts by Western blotting with rat mAb 6G3 directed against the C terminus of the regulatory A subunit (30). The detection of the C and Bα subunits by Western blotting was carried out as described (30). Because PP2A is highly conserved between human and *Xenopus* (31), antibodies directed against the A, B, and C subunits of the human PP2A crossreact strongly with their *Xenopus* counterparts. Signals were detected by enhanced chemiluminescence (Amersham). PP2A core enzyme was depleted from extract with rat mAb 5H4 coupled to protein G Sepharose, by using the same procedure as for mAb 6F9. mAb 5H4 recognizes core enzyme but not holoenzyme (30).

DNA Replication Assay. DNA replication was measured by incorporation of [α-^{32}P]dCTP into DNA. In the nuclear system, egg cytosol was supplemented with 0.1 vol of purified membranes, an ATP-regenerating system, nocodazole (final concentration was 3.3 μg/ml), and 0.1 mCi/ml [α-^{32}P]dCTP. Sperm from which membranes had been removed was added at a final concentration of 2,000–4,000 sperm per μl. The mixture was incubated in 3-μl aliquots at room temperature for up to 240 min. The reactions were stopped at different time points, analyzed by agarose gel electrophoresis, and quantitated with a Molecular Dynamics PhosphorImager. In the nucleus-free system, sperm was preincubated for 30 min in the mixture described above but without the purified membranes. NPE supplemented with the ATP-regenerating system and [α-^{32}P]dCTP was added to initiate DNA replication.

The amount of C subunit added to rescue DNA replication was calculated based on the observation that PP2A constitutes $\approx$1% of the total protein in the extracts. Egg cytosol generally contains 30–40 mg/ml protein, whereas NPE contains 20–30 mg/ml protein. This amount of C subunit was at least 2-fold more than the amount needed for maximum stimulation of DNA replication. An equal amount of C subunit storage buffer (25 mM Tris, pH 7.5/1 mM EDTA/1 mM DDT/50% glycerol) was added to the controls. The C subunit (a generous gift from Marc Mumby, University of Texas Southwestern Medical Center, Dallas) was purified from bovine heart and was 80% pure.

Chromatin Binding Assay. To analyze the binding of ORC and MCM to chromatin, cold egg-lysis buffer was added to 7 μl of each reaction mixture containing sperm and egg cytosol to a final volume of 50 μl and layered over a 100-μl cushion containing 0.5 M sucrose in 5 $\times$ 44 mm Microfuge tubes (Beckman). Samples were centrifuged at 16,000 $\times$ *g* at 4°C for 40 s in a Beckman E microcentrifuge with a horizontal rotor. The pellet was washed once with egg-lysis buffer, and SDS/PAGE sample buffer was added, followed by Western blot analysis.

RESULTS

PP2A Is Required for Chromosomal DNA Replication in the Presence or Absence of Nuclei. To carry out chromosomal DNA replication, *Xenopus* egg extract is fractionated into a cytosolic and a membrane fraction. When these fractions are mixed with *Xenopus* sperm chromatin, nuclei form and then carry out transport and a single round of chromosomal DNA replication. In this system, the formation of transport-competent nuclei is essential for replication to occur. We found that PP2A represents $\approx$1% of the total protein in egg cytosol (Fig. 1*A*) and that it consists of core enzyme and holoenzyme at a ratio of $\approx$1:1 (Fig. 1*B*), similar to what has been observed in mammalian cells (30). To examine the role of PP2A in DNA replication, we used mAb 6F9 to remove PP2A from egg cytosol. This antibody is directed against the N terminus of the regulatory A subunit of PP2A and precip-

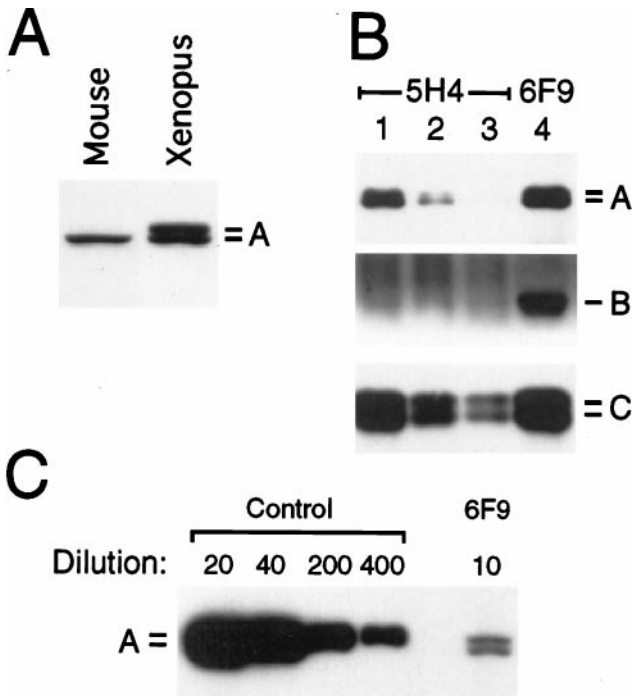

FIG. 1. Quantitation of PP2A in *Xenopus* egg cytosol. (*A*) Equal amounts (5 μg) of protein from 10T1/2 cell cytoplasm and *Xenopus* egg cytosol were analyzed by Western blotting with mAb 6G3, which recognizes the A subunit of PP2A (30). *Xenopus* A subunit migrates as a doublet on SDS/PAGE as described (41). By using the amount of A subunit in 10T1/2 cell extracts (30) and a known amount of purified A subunit (data not shown) as a measure, we estimated that PP2A represents $\approx$1% of total protein in egg cytosol. (*B*) Egg cytosol was precipitated sequentially three times with mAb 5H4 (lanes 1–3), which binds to core enzyme (subunits A and C) but not to holoenzyme (subunits A, B, and C). After removing essentially all the core enzyme, a fourth precipitation was carried out with mAb 6F9 to isolate holoenzyme (lane 4). The result shows that holoenzyme is roughly as abundant in egg extracts as the core enzyme. Identification of all three subunits by Western blotting was performed as described (30). (*C*) PP2A was depleted from egg cytosol with mAb 6F9. To determine the degree of depletion, a serial dilution of undepleted egg cytosol was compared with depleted cytosol by Western blotting with mAb 6G3. A 10-fold dilution of depleted extract contained less A subunit than a 400-fold dilution of undepleted extract. Therefore, the depletion was >97.5%.

itates both holoenzyme and core enzyme (30). After three rounds of depletion with mAb 6F9, >97% of PP2A was removed from egg cytosol (Fig. 1*C*). As shown in Fig. 2 *A* and *B*, removal of PP2A caused almost complete inhibition of DNA replication. The addition of purified C subunit from bovine heart to the PP2A-depleted extract fully restored DNA replication, indicating that the effect of depletion was caused by removal of PP2A. When core enzyme alone was removed with mAb 5H4 (30), no inhibition of DNA replication was observed (data not shown). The finding that simultaneous removal of core enzyme and holoenzyme inhibited replication, whereas removal of core enzyme alone had no effect, indicates that the holoenzyme can perform the function that is essential for replication. Whether the core enzyme can also carry out this function is an open question. When we examined the morphology of the nuclei formed in PP2A-depleted extract, we found that they were smaller than nuclei formed in mock-depleted extract (Fig. 2*C*). The addition of C subunit restored normal nuclear morphology (data not shown). Therefore, it is possible that the effect on replication seen in PP2A-depleted extracts was an indirect consequence of impaired nucleus formation or nuclear transport. Similarly, in a previous study that implicated serine/threonine phosphatases in the stimulation of DNA replication (32), it was impossible to rule out that the effect was caused by problems with nuclear structure.

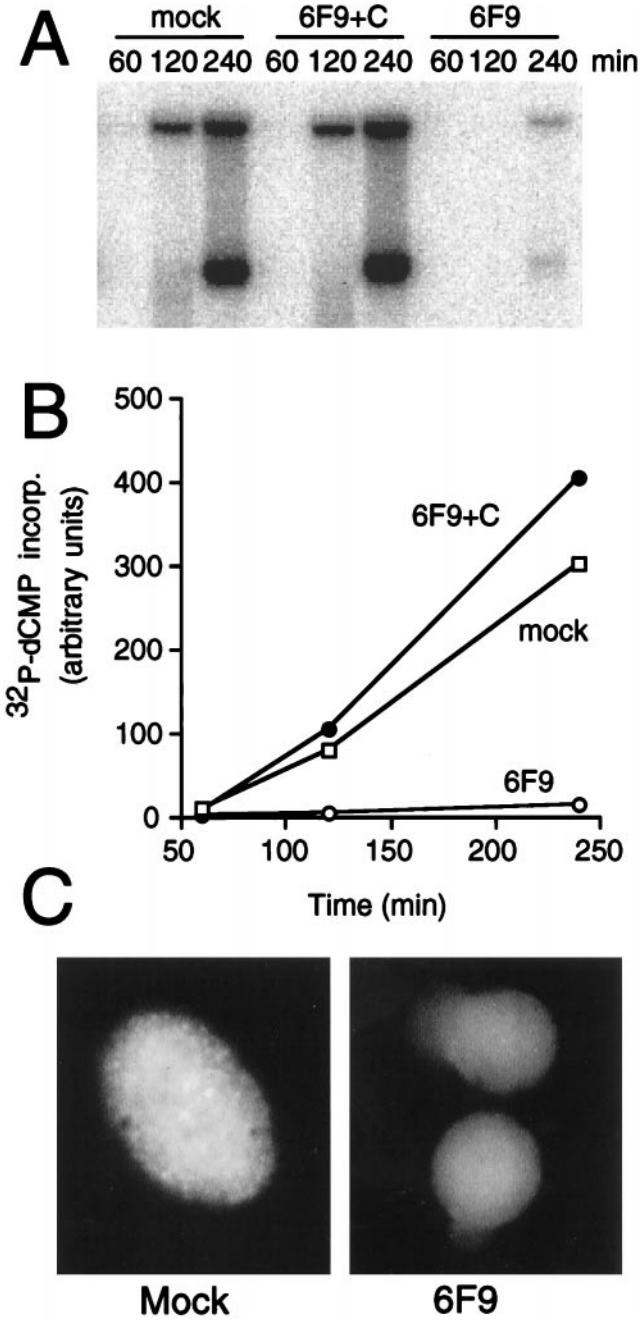

FIG. 2. Inhibition of nuclear DNA replication by removal of PP2A from egg cytosol. Egg cytosol was depleted with either mAb 6F9 or rat IgG. C subunit was added to PP2A-depleted extract at a final concentration of 0.13 μg/μl, which corresponds approximately to the endogenous concentration of PP2A in the extract. Purified membranes, [α-^{32}P]dCTP, and demembranated sperm were added to a concentration of 4,000 sperm per μl. Reactions were stopped at 60, 120, and 240 min, and the DNA were analyzed on a 0.8% agarose gel (*A*) and quantitated with a Molecular Dynamics PhosphorImager (*B*). (*C*) After adding membranes and a 90-min period, the nuclei were analyzed by fluorescence microscopy by using Hoechst dye staining to visualize DNA. Open circles, 6F9 depletion; closed circles, 6F9 depletion plus C subunit; open squares, mock (rat IgG) depletion.

To exclude the possibility of indirect effects, we used a recently developed variation of the egg-extract system that performs DNA replication in the absence of nuclei (29). In this system, demembranated sperm chromatin is first preincubated for 30 min in egg cytosol to allow formation of pre-RCs containing ORC, cdc6, and MCM. Subsequently, instead of adding membranes to form nuclei, a concentrated NPE is added. NPE is extracted from nuclei that were themselves formed in unfractionated *Xenopus* egg extract. The addition of NPE induces pre-RCs to initiate replication, and a complete round of semiconservative DNA replication occurs in the absence of nuclei. It is necessary to form pre-RCs in egg cytosol before adding NPE, because NPE contains an inhibitor that prevents *de novo* pre-RC formation (29). To investigate the role of PP2A in the nucleus-free system, egg cytosol and NPE, which contain similar amounts of PP2A (data not shown), were immunodepleted with mAb 6F9. As shown in Fig. 3*A*, removal of PP2A from both extracts caused strong inhibition of DNA replication in the nucleus-free system. The inhibition was overcome by addition of catalytic C subunit to the NPE. These experiments strongly indicate that PP2A is required directly for DNA replication.

Binding of ORC and MCM to Chromatin in the Absence of PP2A. What step of DNA replication is affected by removal of PP2A? We found that depletion of PP2A from the egg cytosol alone had no effect on DNA replication in the nucleus-free system. This fact was shown by incubating sperm chromatin with mock-depleted or PP2A-depleted egg cytosol for 30 min and purifying the sperm by sedimentation through a sucrose cushion. Undepleted NPE was then added to the chromatin, and DNA replication was measured. The results are shown in Fig. 3*B*. No difference in replication was observed whether PP2A-depleted (Fig. 3*B,* lanes 1 and 2) or mock-depleted egg cytosol (Fig. 3*B,* lanes 3 and 4) was used. In a parallel control experiment, it was shown that the depletion of egg cytosol inhibited DNA replication in the nuclear system (Fig. 3*B*, lanes 5–8). Because NPE contains an inhibitor that prevents *de novo* assembly of pre-RCs (29), this result might suggest that PP2A is not required for pre-RC formation. To investigate this possibility more directly, we determined whether ORC and MCM could bind to sperm chromatin in the absence of PP2A. Sperm chromatin was incubated with PP2A-depleted egg cytosol, purified by centrifugation through a sucrose cushion, and then analyzed by Western blotting. As shown in Fig. 3*C*, removal of PP2A from egg cytosol had no effect on binding of ORC or MCM to the chromatin. This experiment shows that, with regard to the assembly of ORC and MCM, the formation of pre-RCs is unaffected in the absence of PP2A.

PP2A Is Involved in Initiation But Not in Elongation of Replication. We next asked whether PP2A is required for the elongation of previously initiated DNA replication forks. To answer this question, replication was initiated by preincubating

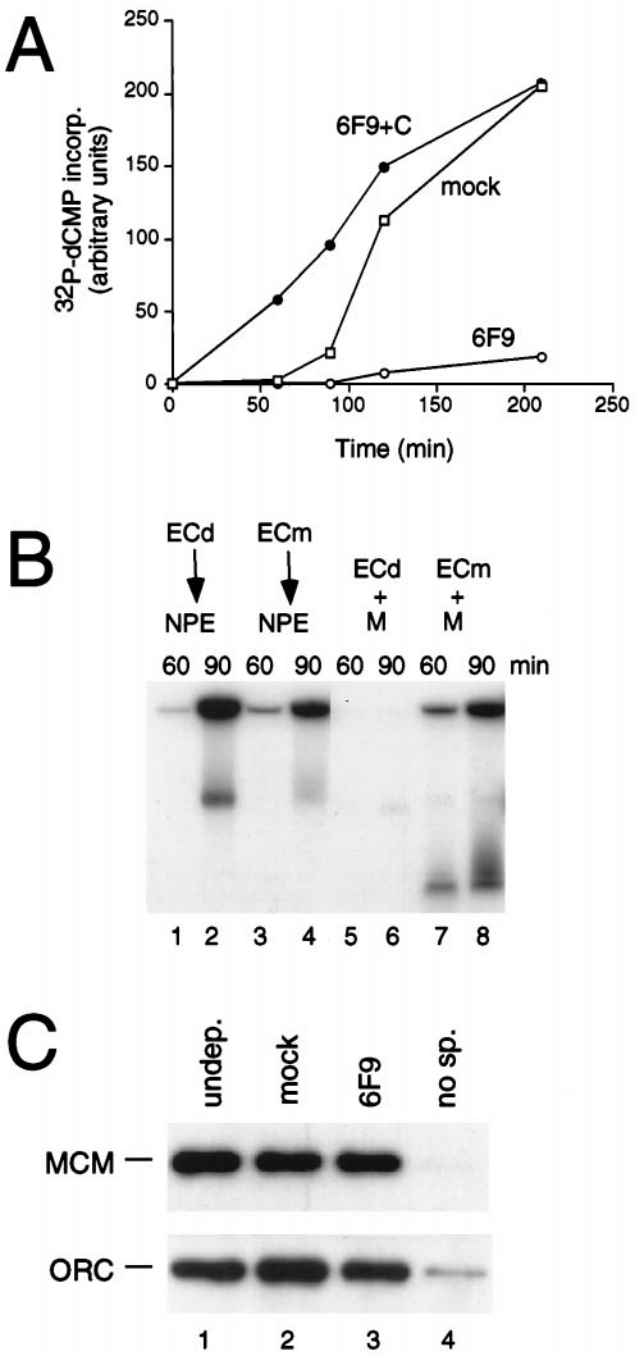

FIG. 3. PP2A is required for DNA replication in the nucleus-free system but not for binding of ORC or MCM to chromatin. PP2A-depleted egg cytosol (2 μl) supplemented with 4,000 sperm per μl was incubated for 30 min. (*A*) Then, 15 μl of NPE, which was 90% depleted of PP2A (data not shown) and supplemented with [α-^{32}P]dCTP, was mixed with the egg cytosol and incubated for 60, 90, 120, and 210 min (open circles). In a parallel experiment, the PP2A-depleted NPE was supplemented with 0.13 μg/μl C subunit (closed circles). In a control experiment, egg cytosol and NPE were mock-depleted with rat IgG, and no C subunit was added (open squares). (*B*) Sperm chromatin (final concentration of 2,000 sperm per μl) was incubated in PP2A-depleted (lanes 1 and 2) or mock-depleted (lanes 3 and 4) egg cytosol. After 30 min, the chromatin was recovered by centrifugation through a sucrose cushion. NPE supplemented with [α-^{32}P]dCTP was added, and replication was measured after 60 and 90 min. (*B*) PP2A-depleted (lanes 5 and 6) or mock-depleted (lanes 7 and 8) egg cytosol was supplemented with purified membranes and [α-^{32}P]dCTP. Sperm (final concentration of 2,000 sperm per μl) was added, and replication was measured after 60 and 90 min. More (3×) sperm chromatin was loaded in lanes 1–4 than in lanes 5–8. (*C*) After a 30-min incubation of 4,000 sperm per μl in egg cytosol, sediment from sperm chromatin from 7 μl was collected in a sucrose cushion and analyzed by Western blotting with antibodies against XORC2 (42) and XMCM3 (43). Lane 1, undepleted egg cytosol; lane 2, mock-depleted egg cytosol; lane 3, 6F9-depleted egg cytosol; and lane 4, undepleted egg cytosol lacking sperm.

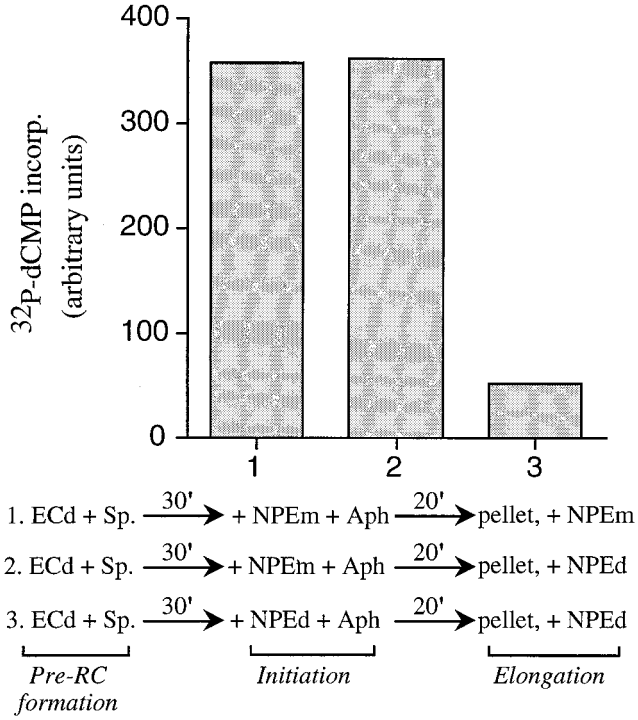

FIG. 4. PP2A is required for initiation but not for elongation of DNA replication. (Column 1) PP2A-depleted egg cytosol (EC; 4 μl) containing 2,000 sperm per μl was incubated for 30 min. Then, 12 μl of mock-depleted NPE containing 50 μg/ml aphidicolin (Aph) was added for 20 min. The chromatin was spun through a sucrose cushion (29), washed, and incubated with 10 μl of mock-depleted NPE and [α-^{32}P]dCTP. Replication was measured after 50 min, when the replication rate was still within the linear range. (Column 2) The incubation was performed as for column 1, except that PP2A-depleted NPE was used for the elongation reaction. (Column 3) The incubation was performed as for column 2, except that PP2A-depleted NPE was also used for the initiation step. The amount of replication observed in columns 1 and 2 was 25% of the replication observed in nuclei (data not shown), which are known to replicate 100% of the input DNA (27). ECd, PP2A-depleted egg cytosol; NPEd, PP2A-depleted NPE; NPEm, mock-depleted NPE.

sperm chromatin in egg cytosol and then adding NPE. The NPE used at this step was supplemented with aphidicolin, a specific inhibitor of elongation, to stall replication forks after they had initiated. The chromatin was then isolated and resuspended in fresh NPE (lacking aphidicolin) that was PP2A-depleted or mock-depleted, and elongation was measured. As shown in Fig. 4, column 2, removal of PP2A from NPE had no inhibitory effect on replication relative to the mock-depleted control (column 1), indicating that PP2A is not required for elongation. However, when PP2A was also removed from the NPE containing aphidicolin, replication was strongly inhibited (column 3). Because initiation takes place during this step, we conclude that PP2A is an initiation factor.

DISCUSSION

We have shown that in *Xenopus* egg extracts, PP2A is essential for chromosomal DNA replication. Because PP2A is not required for elongation of previously engaged replication

forks, we infer that PP2A is required for the initiation of DNA synthesis. An important part of initiation is the ordered binding of ORC, cdc6, and MCM to the chromatin (33). In PP2A-depleted extracts, normal amounts of ORC and MCM bind to chromatin, and it is possible to infer that cdc6 also binds, because its binding is a prerequisite for the loading of MCM. However, we cannot rule out that other aspects of pre-RC structure might be defective in the absence of PP2A. For example, it is possible that ORC, cdc6, or MCM must be dephosphorylated by PP2A to render the pre-RC competent for initiation or that currently unknown components of the pre-RC only bind in the presence of PP2A. Alternatively, later steps of initiation might be affected by the loss of PP2A. For example, it may be that the single-stranded DNA binding protein RPA or the initiation factor cdc45 (34, 35) must be dephosphorylated to function. Finally, it is possible that PP2A is required to activate cyclin-dependent kinase 2/cyclin E or cdc7/dbf4, the protein kinases that stimulate passage through the G_1/S transition.

Because PP2A antagonizes cdc2/cyclin B in *Xenopus* egg extracts (see below), there is a possibility that removal of PP2A inhibits DNA replication indirectly by inducing a mitotic state. However, this possibility is very unlikely, because the extracts used in our experiments were derived from activated eggs, which contain little or no cyclin B. In addition, several observations indicate directly that the extracts were not in mitosis. First, nuclei formed in the absence of PP2A. Second, in the PP2A-depleted extract lacking membranes, there was no condensation of the sperm chromatin (data not shown). Third, both ORC and MCM bound to chromatin in PP2A-depleted extracts, whereas neither protein binds to chromatin in mitosis (33, 36). Therefore, we conclude that PP2A normally plays a role in initiation of DNA replication during interphase.

The question arises as to whether PP2A has a similar function in other eukaryotic organisms. There is evidence from Lin and Arndt (37) that PP2A activity is required for the G_1/S transition in *Saccharomyces cerevisiae*. Those authors generated a temperature-sensitive mutant of the PP2A catalytic subunit (pph21–102). They found that when asynchronous cultures of this mutant were shifted to the restrictive temperature, most cells were blocked in G_2. However, when pph21–102 cells were blocked in G_1 with α-factor at the permissive temperature, then released from the block, and immediately shifted to the restrictive temperature, they remained arrested. Furthermore, when the mutant cells were shifted at a later time after being released from the block, and presumably after having initiated DNA replication, they recovered from the α-factor arrest and completed S phase. These results suggest to us that PP2A is required for initiation of DNA replication in *S. cerevisiae*. (To our knowledge, similar observations have not been reported for *Schizosaccharomyces pombe*). Together with our own data, these results indicate that the initiation of eukaryotic DNA replication in general is controlled positively by dephosphorylation, in addition to being controlled positively by phosphorylation. Our results also imply that protein kinases not only activate but also inactivate initiation and that the inactivating protein kinases are counteracted by PP2A.

Another important question is which form of PP2A controls DNA replication in *Xenopus* egg extract. We have shown that egg extract contains similar amounts of holoenzyme and core enzyme, as was found in various mammalian tissue culture cells (30) and in heart tissue (unpublished results). In neither case was there evidence for the presence of free catalytic C subunit. The fact that selective depletion of core enzyme had no effect on DNA replication implies that holoenzyme alone can support replication. Because we cannot remove holoenzyme selectively, we do not know whether core enzyme alone can also support replication. It would be interesting to remove all forms of PP2A with 6F9 antibodies and then return either core enzyme or holoenzyme purified from *Xenopus* eggs. In addition, we would like to find out how many different forms of B subunits (B, B′, and B′′) are present in egg extract and whether any particular form is involved in DNA replication. It is tempting to speculate that in intact cells, a specific form of PP2A may be required either because of its specific subcellular localization (e.g., the nucleus) or its unique substrate specificity. Our finding that exogenous free C subunit supports DNA replication, although this form does not exist in egg extract, indicates that, functionally, C subunit can replace the holoenzyme and perhaps the core enzyme (14, 25) in supporting DNA replication. This finding is not surprising, because it is known that, depending on the substrate, the C subunit can have overlapping activities with the core enzyme or the holoenzyme.

PP2A plays an important role at the G_2/M transition in fission yeast (*S. pombe*), budding yeast (*S. cerevisiae*), and in *Xenopus* egg extract (reviewed in ref. 14). Interestingly, whereas in fission yeast PP2A inhibits entry into mitosis (38), PP2A plays a positive role in budding yeast (37). It was also shown that the B subunit of PP2A is required for proper functioning of the spindle-assembly check point in *S. cerevisiae* (39). In *Xenopus* egg extract, PP2A functions as an inhibitor of cdc2/cyclin B activation (reviewed in ref. 40), presumably by controlling the phosphorylation state at the regulatory sites Thr-161 and Tyr-15 of cdc2. Lee *et al.* (41) purified and characterized the inhibitory form of PP2A from *Xenopus* egg extract and showed that it was holoenzyme containing the Bα subunit. This subunit was also identified in our experiments, suggesting that the same form of holoenzyme that inhibits entry into mitosis may also control initiation of DNA replication. However, our data do not exclude the possibility that different forms of holoenzyme control these two processes, because we only used antibodies against the Bα subunit. Considering the large number of available regulatory B subunits, it would not be surprising if the cell used different B subunits in combination with the same core enzyme to control different phases of the cell cycle.

We thank Marc Mumby for his gift of purified C subunit. This work was supported by Public Health Service Grants CA-36111 (to G.W.), RO1GM33523 (to J.N.), and 1F32GM17980-01 (to J.W.).

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