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Evidence for Sequential Action of cdc7 and cdk2 Protein Kinases during Initiation of DNA Replication in Xenopus Egg Extracts*

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To investigate how the protein kinase cdc7 stimulates DNA replication in metazoans, a soluble cell-free replication system derived from *Xenopus* eggs was used. DNA was incubated in egg cytosol to form prereplication complexes and then in nucleoplasmic extract to initiate DNA synthesis. We find that cdc7 is greatly enriched in nucleoplasmic extract and that this high concentration is essential for efficient DNA replication, supporting previous models that the nucleus activates replication indirectly by sequestering essential components. cdc7 binds to chromatin at the G_1/S transition before initiation occurs, and it dissociates from chromatin as S phase progresses. The chromatin association of cdc7 requires chromatin-bound MCM. In turn, cdc7 is required to load the initiation factor cdc45 onto the DNA. Finally, efficient replication is observed when chromatin is exposed first to cdc7 and then to cdk2 but not when it is exposed to cdk2 before cdc7. Therefore, the cdc7- and cdk2-dependent initiation steps can be separated, indicating the existence of a novel, stable initiation intermediate. Moreover, the data suggest that cdk2 can only act after cdc7 has executed its function.

Eukaryotic cells regulate the initiation of DNA replication via the ordered assembly and disassembly of replication complexes at origins of replication (1). During the G_1 phase of the cell cycle, prereplication complexes (pre-RCs)[1] are assembled through the sequential binding of cdc6 and the MCM complex with a chromatin-bound origin recognition complex (ORC). At the G_1/S transition, pre-RCs are activated for replication by an S phase-specific cyclin-dependent kinase (cdk2) and a second cdk-like kinase, cdc7. Activation of pre-RCs causes their disassembly through the loss of MCM from the origin. *De novo* binding of MCM to chromatin is inhibited by S and M phase-specific cdks, such that new pre-RCs cannot be formed until cdk activity is destroyed in mitosis. As a result, replication can initiate only once from pre-RCs that were assembled during the G_1 phase of the cell cycle.

At the G_1/S transition, the initiation factor cdc45 associates with the pre-RC to generate a preinitiation complex. In yeast, this event appears to require both the action of cdk and cdc7/dbf4 (2, 3), and temperature shift experiments suggest that cdk exerts its function before cdc7 (4). In *Xenopus* egg extracts, cdc45 loading has been shown to require cdk2 (5, 6). After cdc45 binding, the next detectable event in initiation is the unwinding of the origin, a process that requires the eukaryotic single-stranded DNA-binding protein, RPA (6, 7, 43). Although the helicase that unwinds the DNA is not known, there is mounting evidence it may be MCM (8–11). Once the origin has been unwound sufficiently, DNA polymerase α, the presumptive initiating DNA polymerase, is recruited to the origin, and DNA synthesis can begin (6, 7, 43).

cdc7 was first isolated in budding yeast, and its activity was shown to depend on a regulatory subunit, dbf4, which is expressed in mid-G_1 and S phase (reviewed in Ref. 12). The requirements for cdc7 and dbf4 in replication initiation can be completely bypassed by a point mutation in MCM5 called *bob1* (13), and MCM2 mutations are suppressed by mutations in dbf4 (14). Moreover, several MCM subunits are phosphorylated by cdc7 *in vitro* and *in vivo* (reviewed in Ref. 12). These observations strongly suggest that MCMs are positive targets of cdc7 phosphorylation. In contrast to cdc7/dbf4, little is known about what component(s) of the replication machinery must be phosphorylated by cyclin-dependent kinases to stimulate initiation of DNA replication.

This paper examines the roles of *Xenopus* cdc7 and cdk2 in the initiation of DNA replication. A *Xenopus laevis* cdc7 homolog (xCdc7) has been isolated (15) and shown by antibody interference experiments to be required for DNA replication in *Xenopus* egg extracts (16). cdk2/cyclin E is the only cdk with significant activity in interphase *Xenopus* egg extracts, and it appears to be the only cdk necessary to support DNA replication in this system (17, 18). To facilitate the analysis of these kinases, we are using a modification of *Xenopus* nuclear assembly extracts in which DNA replication occurs in the absence of nuclei (19). In this system, replication is initiated in two steps. First, demembranated sperm chromatin is incubated with membrane-free egg cytosol to form pre-RCs. Second, a nucleoplasmic extract (NPE) is added, which initiates replication, and a complete round of semi-conservative DNA replication takes place (19). This system has been used to further define the role of xCdc7 in replication initiation and to understand its functional relationship to cdk2.

EXPERIMENTAL PROCEDURES

DNA Replication and Chromatin Binding Assays—Extract preparation and replication assays were carried out as described (19). To inhibit initiation, purified glutatione *S*-transferase-tagged $p27^{Kip}$ (20, 21) was preincubated with extracts for 10 min at a final concentration of 1 μM. To isolate chromatin (22), up to 12 μl of extract containing 10,000 sperm/μl was mixed with 70 μl of egg lysis buffer (ELB; 250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Hepes, pH 7.7) containing 0.2%

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[1] The abbreviations used are: pre-RC, prereplication complex; ORC, origin recognition complex; MCM, minichromosome maintenance; NPE, nucleoplasmic extract; cdk, cyclin-dependent kinase; ELB, egg lysis buffer.

Triton X-100 and then centrifuged through a sucrose cushion. The chromatin pellet was washed with 200 μl of ELB.

Immunological Methods—To deplete proteins from egg cytosol or NPE, 1 volume of extract was incubated for 3 h with 0.2 volumes of protein A-Sepharose fast flow (Amersham Pharmacia Biotech) that had been bound to 0.6 volumes of xCdc7 antiserum (6), xMcm7 antiserum (6), or the appropriate preimmune serum. Extract was harvested, and the procedure was repeated once. Western blotting was performed using antisera at 1:5000 dilution against xCdc7 (6), xMcm3 (23), xOrc2 (22), xCdc45 (6), and the 34-kDa subsunit of xRpa (6).

RESULTS

The Rate of DNA Replication Is Regulated by the Concentration of cdc7—We previously showed that immunodepletion of egg cytosol and NPE with an antibody raised against xCdc7 protein prevented replication initiation (6). To better understand how xCdc7 contributes to replication initiation, egg cytosol and NPE were immunoblotted with anti-xCdc7 antibody. The concentration of xCdc7 in NPE was 10–20 times higher than in egg cytosol (Fig. 1*A*, compare *lane 1* with *lanes 2–4*), indicating that xCdc7 localizes to the nucleus.

It was of interest to determine whether xCdc7 must be supplied by egg cytosol, NPE, or both to drive replication. When both extracts were depleted of xCdc7 (Fig. 1*A*, *lanes 5* and *6*), replication was eliminated relative to control extracts that were depleted with preimmune serum (Fig. 1*B*, compare *squares* and *diamonds*). When egg cytosol but not NPE was depleted of xCdc7, replication was normal (Fig. 1*B*, *circles*). However, when NPE but not egg cytosol was depleted of xCdc7, the rate of replication was reduced 5–6-fold (Fig. 1*B*, *triangles*). Therefore, although egg cytosol-derived xCdc7 can support significant levels of DNA replication, NPE-derived xCdc7 is essential to achieve rates of DNA replication that are similar to what is observed in nuclei and in embryos.

To ask whether the high concentration of xCdc7 present in NPE is important for efficient DNA replication, we incubated sperm chromatin in mock depleted egg cytosol to form a pre-RC. We then added mock depleted and cdc7-depleted NPE in different ratios to determine what concentration of nuclear xCdc7 is necessary to stimulate efficient DNA replication. Reactions in which the xCdc7-depleted NPE was mixed in a 10:1 or 1:1 ratio with mock depleted NPE (Fig. 1*C*, *diamonds* and *circles*) replicated better than reactions containing only cdc7-depleted NPE (Fig. 1*C*, *squares*). However, the most efficient replication was obtained when only mock depleted NPE was used (Fig. 1*C*, *triangles*). Therefore, a full complement of NPE-derived xCdc7 is necessary to achieve efficient DNA replication, indicating that this protein kinase must be present at high concentration to function efficiently in *Xenopus* egg extracts.

MCMs Are Required to Recruit xCdc7 to Chromatin—cdc7 and dbf4 have been shown to bind chromatin in budding yeast (24, 25). We therefore tested whether xCdc7 binds to chromatin in egg extracts. Very low levels of xCdc7 bound to chromatin in egg cytosol (Fig. 2, *lane 1*; see also Fig. 3*A*, *lane 1*), and much higher amounts bound within 5 min after addition of NPE (Fig. 2, *lane 2*, and Fig. 3*A*, *lane 5*). As replication proceeded, xCdc7 was gradually lost from the chromatin with similar kinetics as xMcm3 (Fig. 2, *lanes 2–5*, compare xCdc7 and xMcm3 panels). When DNA replication was inhibited with aphidicolin, neither MCM nor cdc7 were displaced (Fig. 2, *lanes 10–13*). When initiation of replication was blocked by the cdk2 inhibitor $p27^{Kip}$ (20, 21), MCM again was not displaced (Fig. 2, *lanes 6–9*). In the presence of $p27^{Kip}$, the amount of xCdc7 that loaded onto chromatin at the G_1/S transition was slightly less, and interestingly, its mobility became retarded over time. The significance of this observation is not clear at present. Together, the above results show that there is a dramatic increase

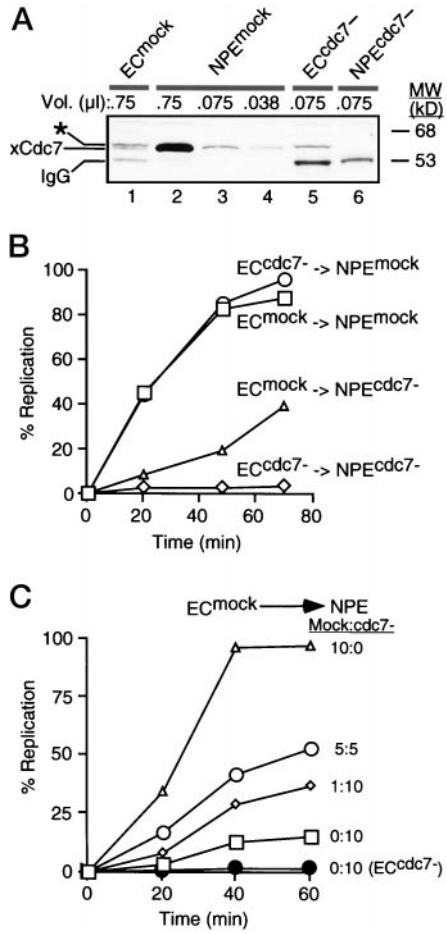

FIG. 1. **The high concentration of xCdc7 present in NPE is essential for efficient DNA replication.** *A*, xCdc7 is ~20 times more concentrated in NPE than in egg cytosol. 0.75 μl of mock depleted egg cytosol (*EC*) (*lane 1*), 0.75, 0.075, and 0.0375 μl of mock depleted NPE (*lanes 2–4*, respectively), 0.75 μl of xCdc7-depleted egg cytosol (*lane 5*), or 0.75 μl of xCdc7-depleted NPE (*lane 6*) were separated through a 10% SDS-polyacrylamide gel and immunoblotted with xCdc7 antiserum. *IgG*, contamination from depletion procedure; *, cross-reacting protein. *B*, sperm chromatin (final concentration, 10,000/μl) was incubated for 30 min with mock depleted (*squares* and *triangles*) or xCdc7-depleted egg cytosol (*circles* and *diamonds*) and then supplemented with 2 volumes mock depleted (*circles* and *squares*) or xCdc7-depleted NPE (*triangles* and *diamonds*) containing [α-^{32}P]dATP, and replication was measured 20, 48, and 70 min after NPE addition. *C*, sperm chromatin was incubated for 30 min with xCdc7-depleted (*filled circles*) or mock depleted egg cytosol (*open triangles*, *circles*, *diamonds*, and *squares*) and then supplemented with 2 volumes total of mock depleted and xCdc7-depleted NPE mixed in different ratios as indicated in the figure. The NPE also contained [α-^{32}P]dATP, and replication was measured 20, 40, and 60 min after NPE addition.

in the level of cdc7 binding to chromatin upon addition of NPE but before initiation occurs, and xCdc7 is displaced from chromatin during S phase in a process that appears to require movement of the replication fork. It is noteworthy that the amount of RPA that loads onto chromatin is dramatically enhanced in the presence of aphidicolin (Fig. 2, compare *lanes 2–5* with *10–13*), consistent with our previous observation that aphidicolin induces massive DNA unwinding (6).

MCMs are likely targets of cdc7 phosphorylation (12) and are therefore attractive candidates for proteins that might recruit cdc7 to chromatin. To test this, the replication inhibitor geminin was used because it blocks the loading of MCMs onto chromatin without affecting ORC or cdc6 binding (26). Low levels of xCdc7 were bound in egg cytosol, and higher levels were bound after addition of NPE (Fig. 3*A*, *lanes 1* and *5*). In the presence of geminin, binding of both xMcm3 and xCdc7 to

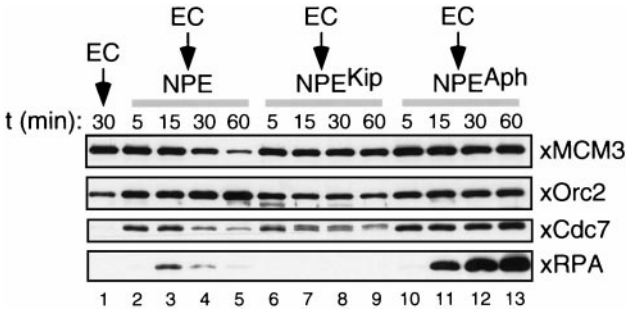

FIG. 2. **xCdc7 binds to chromatin before replication initiation, and it is displaced from chromatin during S phase.** Sperm chromatin was incubated with egg cytosol (*EC*, *lane 1*) or egg cytosol followed by NPE (*lanes 2–5*), NPE containing $p27^{Kip}$ (*lanes 6–9*), or NPE containing 50 μg/ml aphidicolin (*lanes 10–13*). After the indicated time, chromatin was isolated through a sucrose cushion and blotted with antibodies against xMCM3, xOrc2, xCdc7, or xRPA.

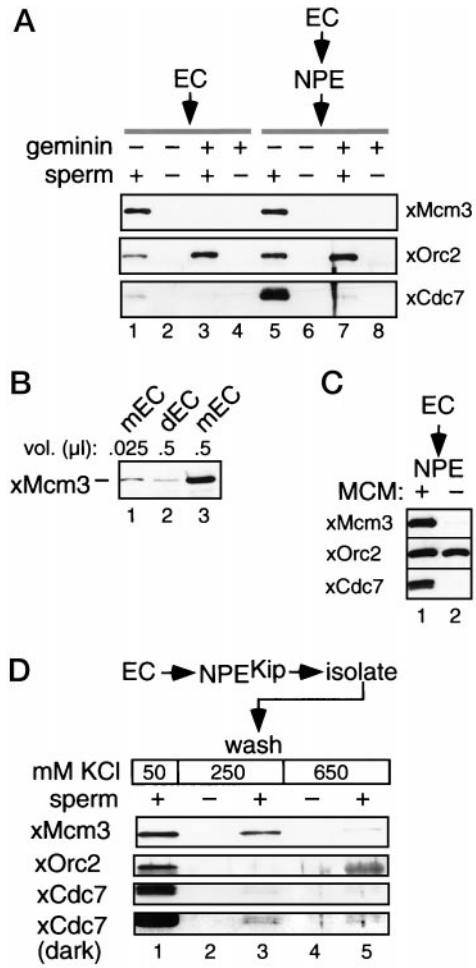

FIG. 3. **XCdc7 binding to chromatin is MCM-dependent and salt-sensitive.** *A*, sperm chromatin was incubated with egg cytosol (*EC*) containing (*lanes 3*, *4*, *7*, and *8*) or lacking 100 nM his-tagged human geminin (*lanes 1*, *2*, *5*, and *6*). Chromatin was isolated after 30 min (*lanes 1–4*) or after a further 30 min incubation with NPE (*lanes 5–8*) and blotted with antibodies against xMcm3, xOrc2, and xCdc7. Controls lacking sperm were included (*lanes 2*, *4*, *6*, and *8*) *B*, 0.5 μl of egg cytosol depleted with anti-xMcm7 antibody (*lane 2*), and 0.5 μl (*lane 3*) or 0.025 μl (*lane 1*) of mock depleted egg cytosol were immunoblotted with anti-xMcm3 antibodies. *C*, sperm chromatin was incubated with mock depleted egg cytosol followed by NPE containing $p27^{Kip}$ to prevent replication-mediated removal of MCM (*lane 1*) or xMcm7-depleted egg cytosol followed by NPE (*lane 2*). After 30 min in NPE, chromatin was isolated and blotted for xMCM3, xOrc2, or xCdc7. *D*, sperm chromatin was incubated in egg cytosol for 30 min and then supplemented with NPE containing $p27^{Kip}$. Chromatin was diluted with ELB containing 0.2% Triton X-100 (ELB/TX; *lane 1*) or ELB/TX containing 250 mM final concentration of KCl (*lanes 2–5*) and centrifuged through a sucrose cushion. The chromatin pellet was washed with ELB (*lanes 1–3*) or ELB/TX containing 650 mM (final concentration) of KCl (*lanes 4* and *5*). Samples 4 and 5 were washed once more with ELB. The pellet was immunoblotted with antibodies against xMcm3, xOrc2, or xCdc7. The *lower cCdc7 panel* was exposed for a long time.

chromatin was almost completely abolished (Fig. 3*A*, *lanes 3* and *7*), whereas binding of ORC was enhanced, as previously reported (Fig. 3*A*, *lanes 3* and *7*) (26). Identical results were obtained when NPE containing $p27^{Kip}$ was used to block replication initiation (data not shown). Because it is not known whether geminin affects the binding of MCMs directly or through another factor such as cdt1 that is required for MCM binding (27), we used antibodies against xMcm7 to remove the MCM complex from egg cytosol. Depletion was at least 95% efficient (Fig. 3*B*, compare *lanes 1* and *2*). Sperm chromatin was incubated with MCM depleted egg cytosol for 30 min and was then supplemented with undepleted NPE. Because NPE contains inhibitors of MCM binding (19), it was not necessary to deplete MCMs from NPE. Indeed, even after the addition of undepleted NPE, there was no MCM bound to chromatin (Fig. 3*C*, *lane 2*). Under these conditions, xCdc7 binding to chromatin was much less than the control, which contained chromatin-bound MCM (Fig. 3*C*, compare *lanes 1* and *2*). Together, these data indicate that the MCM complex is essential to recruit xCdc7 to chromatin.

The stability of chromatin-bound xCdc7 was also examined (Fig. 3*D*). Sperm chromatin was incubated in egg cytosol for 30 min followed by an additional 30 min incubation with NPE. $p27^{Kip}$ was included with the NPE to prevent removal of cdc7 from the chromatin by replication. Like ORC (28), xCdc7 was almost completely eluted from chromatin by 250 mM KCl (Fig. 3*D*, compare *lanes 1* and *3*). A small amount of xCdc7 remained bound to chromatin under these washing conditions (Fig. 3*D*, *lane 3*, dark exposure). However, this xCdc7 remained bound even after exposure of the chromatin to 650 mM KCl, a treatment that displaces MCM from chromatin (Fig. 3*D*, *lane 5*). Because xCdc7 recruitment to chromatin is MCM-dependent (Fig. 3*C*), the residual xCdc7 bound to chromatin after exposure to high salt is likely bound to nonorigin DNA and therefore nonfunctional. The salt displacement of xCdc7 is important for the interpretation of chromatin-transfer experiments shown below.

xCdc7 Is Required to Load xCdc45 onto Chromatin—We previously showed that xCdc7 and xCdc45 are required for origin unwinding (6). To determine whether xCdc7 is required for binding of xCdc45 to DNA at the G_1/S transition, sperm chromatin was incubated sequentially with xCdc7-depleted egg cytosol and xCdc7-depleted NPE, isolated, and blotted for xCdc45. Compared with mock depletion, xCdc7 depletion caused a dramatic reduction in the amount of xCdc45 that loaded onto chromatin (Fig. 4, compare *lanes 1* and *3*). The reduction is equivalent to that observed when cdk2 is inactivated by $p27^{Kip}$ (Fig. 4, *lane 2*, and Refs. 5 and 6). Thus, both cdk2 and xCdc7 are required to recruit xCdc45 to origins at the G_1/S transition. As expected, given its requirement for unwinding (6), xCdc7 is also necessary to load RPA onto the chromatin (Fig. 4, *lane 3*).

The xCdc7 and cdk-dependent Initiation Steps Are Separable—As seen from the data in Fig. 4, the execution points of cdk2 and xCdc7 are identical, because neither kinase is required for pre-RC assembly, and both are needed to recruit xCdc45. The question arises of whether xCdc7 and cdk2 exert their effects on pre-RCs in a defined order, and if so, which kinase acts first. It was first tested whether xCdc7 could exert its function before cdk2 (Fig. 5*A*, *sequence 2*; see Fig. 5*B* for

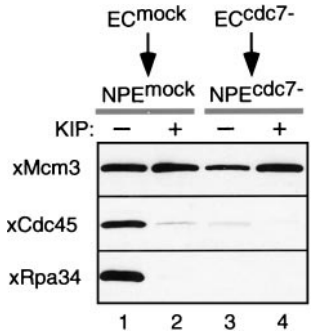

FIG. 4. **XCdc7 is required for recruitment of xCdc45 to chromatin.** Sperm chromatin was incubated with mock depleted (*lanes 1* and *2*) or xCdc7-depleted egg cytosol (*EC*, *lanes 3* and *4*). After 30 min, mock depleted (*lanes 1* and *2*) or xCdc7-depleted NPE supplemented with 50 μg/ml aphidicolin and containing (*lanes 2* and *4*) or lacking (*lanes 1* and *3*) $p27^{Kip}$ was added. After 30 min, chromatin was isolated and immunoblotted with xMcm3, xCdc45, or xRPA antibodies.

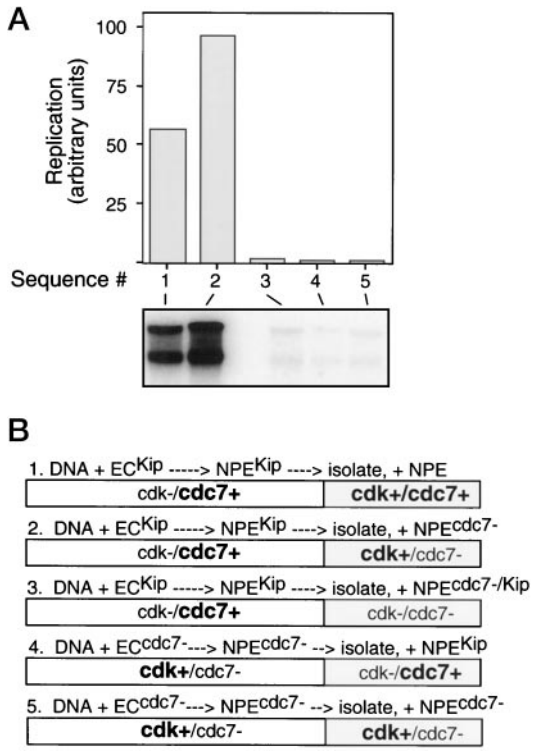

FIG. 5. **The xCdc7- and cdk2-dependent replication initiation steps can be experimentally separated.** *A*, sperm was incubated for 30 min with 2 μl of xCdc7-depleted (*sequences 4* and *5*) or mock depleted egg cytosol containing $p27^{Kip}$ (*sequences 1–3*). After 30 min, 4 μl of xCdc7-depleted NPE (*sequences 4* and *5*) or mock depleted NPE containing $p27^{Kip}$ (*sequences 1–3*) was added. After 30 min, the reaction was diluted with ELB containing 250 mM KCl and 0.2% Triton X-100 and isolated. The pellet was washed with ELB and then supplemented with 5 μl mock depleted NPE (*sequence 1*), xCdc7-depleted NPE (*sequences 2* and *5*), xCdc7-depleted NPE containing $p27^{Kip}$ (*sequence 3*), or mock depleted NPE containing $p27^{Kip}$ (*sequence 4*), and replication was measured in the presence of [α-^{32}P]dATP for 60 min. As observed previously (19), maximum replication efficiency of isolated chromatin was typically ~30%. *B*, schematic representation of experiments shown in *A*. *EC*, egg cytosol.

schematic representations of experiments performed in Fig. 5*A*). To allow the xCdc7-dependent step to occur in the absence of cdk2 activity, sperm chromatin was incubated for 30 min with egg cytosol containing $p27^{Kip}$ and then for another 30 min following the addition of NPE containing $p27^{Kip}$. It was necessary to use both egg cytosol and NPE to execute the cdc7 step because xCdc7 is only maximally active when supplied by NPE (Fig. 1*B*). The chromatin was then isolated through a sucrose cushion in the presence of 250 mM KCl and incubated in xCdc7-depleted NPE lacking $p27^{Kip}$ to supply cdk2 activity in the absence of xCdc7. [α-^{32}P]dATP was included in this second incubation and replication was measured after 60 min. This chromatin replicated (Fig. 5*A*, *sequence 2*), and the efficiency of replication was at least as good as that of chromatin which went through a similar set of manipulations but where both kinases were allowed to function at the same time (Fig. 5*A*, *sequence 1*). The replication observed in sequence 2 is unlikely because of chromatin-mediated transfer of xCdc7 from the first to the second extract because the chromatin was isolated in the presence of 250 mM KCl, which reduces chromatin-bound xCdc7 to background levels (Fig. 3*D*). Furthermore, controls show that xCdc7 was functionally depleted from extracts (Fig. 5, *sequence 5*) and that when $p27^{Kip}$ was present in all incubations, no replication took place (Fig. 5, *sequence 3*). These results strongly argue that in *Xenopus* egg extracts, the xCdc7 and cdk2 initiation steps are separable and that xCdc7 can exert its function before cdk2.

Evidence That cdk2 Cannot Execute Its Function before cdc7—We next asked whether replication occurs when chromatin is exposed to the two protein kinases in the reverse order (cdk2 first, cdc7 second). To supply cdk2 activity in the absence of xCdc7, sperm chromatin was incubated for 30 min in xCdc7-depleted egg cytosol and then for a further 30 min after addition of xCdc7-depleted NPE. The incubation in xCdc7-depleted NPE was necessary to execute the cdk2 step because like xCdc7, cdk2/cyclin E must be supplied by NPE (19).[2] After the incubations with cdk2, the chromatin was isolated in the presence of 250 mM KCl and supplemented with mock depleted NPE containing $p27^{Kip}$ and [α-^{32}P]dATP. This second incubation was intended to allow the cdc7-dependent step to occur in the absence of cdk2. This series of incubations did not result in any significant DNA replication (Fig. 5, *sequence 4*). The absence of replication in sequence 4 was not due to an effect of $p27^{Kip}$ on elongation because replication of sperm chromatin that had previously initiated replication in the presence of aphidicolin was not affected by the addition of $p27^{Kip}$ (data not shown). Together, these results suggested the possibility that cdk2 cannot exert its function before cdc7.

An alternative explanation for the absence of replication in Fig. 5 (*sequence 4*) was that the product of cdk2 phosphorylation cannot withstand the relatively stringent washing procedure employed during the chromatin transfer. To address this possibility, the same experiment was performed, but the chromatin was isolated in the absence of detergent and using 50 mM instead of 250 mM KCl. Again, no DNA replication was observed (data not shown). To further reduce the possibility of disrupting products of cdk2 phosphorylation, we performed the experiment without the chromatin isolation step (Fig. 6, *sequence 2*). Thus, sperm chromatin was incubated with xCdc7-depleted egg cytosol followed by xCdc7-depleted NPE to allow the cdk2 step to occur. After 20 min in the NPE, $p27^{Kip}$ was added to inactivate cdk2, and after a further 5 min, undepleted NPE containing $p27^{Kip}$ was added to supply xCdc7 activity. This procedure exposed chromatin to cdk2 before xCdc7 without a chromatin isolation step, but there was still no significant DNA replication when compared with a similar sequence in which both kinases are present together (Fig. 6, compare *sequences 2* and *1)*. Indeed, most of the small amount of DNA replication that is observed in sequence 2 is accounted for by residual replication that occurred because of incomplete cdc7 depletion (Fig. 6, *sequence 3*).

In summary, chromatin replicates well when both kinases

[2] J. Walter, unpublished results.

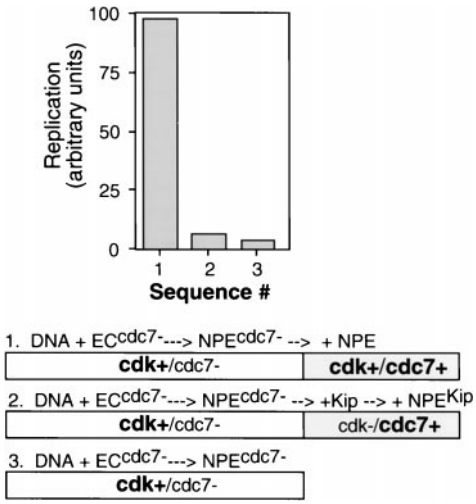

FIG. 6. **DNA replication does not occur when chromatin is exposed to cdk2 and then cdc7, even in the absence of chromatin isolation.** Sperm chromatin (final concentration, 5000/μl) was incubated with 2.5 μl of cdc7-depleted egg cytosol for 30 min and then mixed with 2 volumes of cdc7-depleted NPE. *Sequence 1*, after 25 min, 4 μl of undepleted NPE containing [α-^{32}P]dATP was added to supply cdc7, and replication was measured after further 60 min incubation. *Sequence 2*, the same as sequence 1 except that $p27^{Kip}$ was added 5 min before the addition of undepleted NPE, which also contained $p27^{Kip}$. *Sequence 3*, the cdc7-depleted NPE contained [α-^{32}P]dATP, and replication was measured 60 min after its addition. In each case, the amount of replication per sperm was plotted.

are active simultaneously or when xCdc7 is allowed to act before cdk2 but not when chromatin is exposed to cdk2 before xCdc7. Taken together, the data strongly suggest that xCdc7 must exert its function before cdk2 during replication initiation in *Xenopus* egg extracts.

DISCUSSION

We previously speculated that in *Xenopus* nuclear assembly extracts, the nucleus stimulates DNA replication by acting as a concentrating device for one or more replication factors that do not function at the concentrations present in egg cytosol (19). In agreement with this model, this paper shows that the high concentration of xCdc7 that is present in NPE is essential for efficient DNA replication. However, we cannot at present rule out the possibility that, in addition, nuclear xCdc7 is modified in some fashion that renders it fully active.

Our data show that the recruitment of xCdc7 to chromatin requires the presence of MCM on chromatin. This is consistent with evidence that MCMs are the physiological targets of cdc7 phosphorylation (1), and it suggests that the binding of xCdc7 to chromatin is essential for its function during DNA replication. In further support of this idea, xCdc7 loads onto the DNA before initiation has occurred. This can be seen by the fact that loading is not inhibited when initiation is blocked with $p27^{Kip}$, and loading of xCdc7 precedes the binding of RPA (Fig. 2, compare *lanes 2* and *3* or *10* and *11*), indicating that it occurs before the origin is unwound (6). In contrast, in budding yeast, cdc7 and dbf4 recruitment to the DNA does not require MCM, but rather depends on ORC (29). Moreover, in yeast, cdc7 must be present during pre-RC formation (29). This differs from our observation that efficient replication is achieved when NPE is mixed with pre-RCs that were assembled in egg extracts lacking xCdc7 (Fig. 1*B*, *circles*). Therefore, the mechanism by which cdc7 is recruited to chromatin in yeast and metazoans appears to be different.

cdc45 is an initiation factor whose recruitment to chromatin at the G_1/S transition requires cdk2 (2, 5, 6, 30). Initially, experiments in budding yeast suggested that cdc45 binds chromatin independently of cdc7 (2), but later experiments in the same organism suggested that stable association of cdc45 does require cdc7 (3). This report shows that cdc7 is required for the stable association of cdc45 with chromatin in a metazoan organism. Importantly, we find that like cdk2, xCdc7 is not required for the chromatin binding of MCMs to form pre-RCs (Fig. 3*B*). Based on these criteria, the execution points of xCdc7 and cdk2 during replication initiation are identical.

To ask whether xCdc7 and cdk2 carry out their functions in a defined order, chromatin was transferred between extracts deficient in one or the other kinase. Chromatin exposed first to extract containing only xCdc7 and then to extract containing only cdk2 activity replicated as well as chromatin exposed to extract in which both kinases were active. These results indicate that the ability of xCdc7 to execute its function during replication does not require the simultaneous presence of cdk2 activity or *vice versa*, and this is consistent with observations in yeast (4). The fact that the xCdc7- and cdk2-dependent initiation steps can be separated experimentally further argues that a stable initiation intermediate exists between the pre-RC and the preinitiation complex, but the nature of this intermediate is not clear at present. It is conceivable that in our experiments, some component of the replication machinery, or xCdc7 itself (31), has been preactivated by cdks before the cdc7 step. However, even if this were to be the case, there is clearly an additional requirement for cdk2 after the xCdc7 step has occurred.

Critically, when chromatin was exposed first to cdk2 activity, isolated, and then incubated with extract containing xCdc7, there was no DNA replication. We performed several experiments to address whether this result is due to the product of cdk2 phosphorylation being destroyed during transfer of chromatin between extracts. Most importantly, a sequence in which chromatin is exposed to cdk2 followed by xCdc7 was performed in the absence of chromatin isolation, and still no DNA replication was observed. Although we cannot unequivocally exclude the possibility that in this experiment the cdk2 product was still destroyed, the simplest interpretation of the results is that there is a defined order in which kinases must act during replication initiation in *Xenopus* egg extracts. In this view, xCdc7 must exert its function on pre-RCs before cdk2 can act.

While this work was in progress, Jares and Blow (32) reported an overlapping set of observations. Using nucleus-dependent *Xenopus* egg extracts, these authors found that xCdc7 binds chromatin in an MCM-dependent fashion and that xCdc7 is necessary for cdc45 binding to chromatin. Importantly, although these authors showed that cdc7 *can* exert its function before cdk2, we have provided evidence that it *must* do so.

Our proposal that cdc7 must act before cdk2 in egg extracts has important implications for the regulation of S phase. In metazoans and in yeast, origins initiate replication following a defined temporal order, with some origins firing early in the S phase and others firing late (reviewed in Ref. 33). In yeast, cdc7 and cdks are required for firing of early and late origins (34–36), and this is likely also the case in metazoans. When replication is blocked in the middle of S phase, firing of additional origins is prevented by an "intra-S phase" checkpoint (37–39). It is not known whether sperm chromatin replicating in *Xenopus* extracts obeys an early-late program of origin firing. However, when the activity of cdk2 is inhibited after the first set of initiations has occurred, replication is still extremely efficient (22), indicating that these early initiations support a rapid and complete round of replication. Therefore, it is not unexpected that restricting xCdc7 activity to the beginning of S phase as was done here also results in efficient replication. We further suggest that an initiation mechanism in which cdc7 acts before

cdk2 is not incompatible with an intra-S phase checkpoint, even in somatic cells. It is currently not known why some origins do not fire until late in S phase, but it likely involves higher order chromatin structure or nuclear organization that renders late firing origins refractory to the action of cdk and cdc7 until the appropriate time in S phase. In this view, when a checkpoint is induced during S phase, inhibiting either cdc7 or cdk2 would be sufficient to prevent further origins from firing.

The mechanism and regulation of replication in yeast and metazoans is highly conserved (1, 40). Nevertheless, our findings appear to differ from those made in yeast. Nougarede *et al.* (4) used temperature shift experiments to examine the order in which cdc7 and cdk exert their effects during replication initiation in *Saccharomyces cerevisiae*. They found that cells replicated their DNA when cdk was present before cdc7 but not when cdc7 was present before cdk. This finding is also consistent with earlier observations in *S. cerevisiae* that cdc45 and cdc7 must be present at the same time for replication to initiate (41). However, a recent paper reported that structural changes at yeast origins that are normally dependent on cdc7 can occur in G_1 phase arrested cells that harbor the cdc7-bypass mutation *mcm5/cdc46-bob1* (42). This observation is interesting because it suggests that in *S. cerevisiae*, there is no absolute cdk requirement for at least some of the structural changes at origins that are normally catalyzed by cdc7. More work will be needed to fully resolve to what extent the mechanism of cdc7 action and its relationship to cdk differs between yeast and metazoans.

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