Identification of a Preinitiation Step in DNA Replication That Is Independent of Origin Recognition Complex and cdc6, but Dependent on cdk2

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Abstract. Before initiation of DNA replication, origin recognition complex (ORC) proteins, cdc6, and minichromosome maintenance (MCM) proteins bind to chromatin sequentially and form preinitiation complexes. Using *Xenopus laevis* egg extracts, we find that after the formation of these complexes and before initiation of DNA replication, cdc6 is rapidly removed from chromatin, possibly degraded by a cdk2-activated, ubiquitin-dependent proteolytic pathway. If this displacement is inhibited, DNA replication fails to initiate.

We also find that after assembly of MCM proteins into preinitiation complexes, removal of the ORC from DNA does not block the subsequent initiation of replication. Importantly, under conditions in which both ORC and cdc6 protein are absent from preinitiation complexes, DNA replication is still dependent on cdk2 activity. Therefore, the final steps in the process leading to initiation of DNA replication during S phase of the cell cycle are independent of ORC and cdc6 proteins, but dependent on cdk2 activity.

M ANY of the proteins and regulatory factors involved in the initiation of DNA replication during S phase in eukaryotic cells have been identified and characterized. The multiprotein origin recognition complex (ORC)[1] recognizes and binds to specific DNA sequences in yeast (Bell and Stillman, 1992; Fox et al., 1995; Rao and Stillman, 1995; Donovan and Diffley, 1996). After this, the cdc6 and minichromosome maintenance (MCM) proteins bind sequentially at these same sites to form preinitiation complexes (Coleman et al., 1996; Rowles et al., 1996). The cdc6 protein is essential for DNA replication (Bueno and Russell, 1992; Kelly et al., 1993; Li and Herskowitz, 1993; Liang et al., 1995; Nishitani and Nurse, 1995; Piatti et al., 1995; Cocker et al., 1996; Coleman et al., 1996), and in yeast, turns over rapidly (Nishitani and Nurse, 1995; Piatti et al., 1995; Jallepalli and Kelly, 1996; Muzi-Falconi and Kelly, 1996). The MCM proteins consist of a family of six related proteins, which are also essential for DNA replication (Hennessy et al., 1991; Yan et al., 1991, 1993; Dalton and Whitbread, 1995). During replication these proteins are irreversibly released from DNA and this process is, in part, responsible for limiting replication to a single round each cell cycle (Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995b; Todorov et al., 1995; Coue et al., 1996; Hua et al., 1997).

Using footprinting patterns to examine protein–DNA interactions at origin sequences in yeast, Diffley and coworkers have shown that origins oscillate between two states during the cell cycle. The prereplicative state forms during G1 and requires both ORC and cdc6 proteins (Cocker et al., 1996; Santocanale and Diffley, 1996). During G2 the prereplicative footprint pattern changes to a postreplicative state. Several lines of evidence suggest that this conversion occurs because cdk kinase activates a proteolytic system that degrades cdc6 (Heichman and Roberts, 1996; Jallepalli and Kelly, 1996; Muzi-Falconi et al., 1996; Piatti et al., 1996). Thus it has been proposed that the activation of cdk kinase serves to both block formation of new preinitiation complexes and activate preexisting complexes (Dahmann et al., 1995; Diffley, 1996; Piatti et al., 1996; Wuarin and Nurse, 1996; Hua et al., 1997).

Although it is clear that ORC and cdc6 proteins are required for the formation of preinitiation complexes, it is not equally clear that the continued association of these proteins with DNA is essential for all steps in the initiation process. We have used *Xenopus laevis* egg extracts to investigate this question. Our results strongly suggest that after the assembly of MCM proteins into preinitiation complexes, ORC can be displaced from the complex without inhibiting subsequent initiation steps. Moreover, we show that after assembly of MCM proteins into preinitiation

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1. *Abbreviations used in this paper*: ELB, egg lysis buffer; MCM, minichromosome maintenance; ORC, origin recognition complex.

complexes, the cdc6 protein is rapidly removed from chromatin. The displacement of cdc6 at this time depends on cdk2–cyclin E kinase, which is constitutively active in early embryos and in egg extracts. That the displacement of cdc6 from prereplicative complexes before initiation of DNA replication may occur because of ubiquitin-dependent proteolysis is supported by the observation that addition of R48-ubiquitin inhibits cdc6 displacement. We also find that if cdc6 remains stably associated with chromatin, DNA replication fails to initiate. We also show that after cdc6 displacement, cdk2 kinase is required for at least one more step in the initiation process. Overall, our results suggest that once the MCM proteins have associated with chromatin, ORC and cdc6 proteins may not be essential for the final step leading to initiation of replication at origin sites.

Materials and Methods

Density Substitution

Interphase extract was incubated with 2,000 sperm/μl for 30 min. Cyclin A (100 nM) was added and incubated for another 30 min. After this, membrane was added together with 0.5 mM bromodeoxyuridine (BrdU), 0.5 mM $MgCl_2$, and 10 μCi of [α-^{32}P]dATP, and then the reaction was incubated a further 90 min. After this, 1.5 ml of ice-cold buffer A (50 mM KCl, 50 mM Hepes-KOH, pH 7.4, 5 mM $MgCl_2$, and 1 mM DTT) was added and the sample was incubated for 5 min on ice. The samples were then centrifuged for 5 min in a microfuge, and the resulting nuclear pellet was recovered and resuspended in buffer A. SDS (0.5%) and protease K (0.5 mg/ml; Sigma Chemical Co., St. Louis, MO) were added, and the samples were incubated at 37°C for 1 h. DNA was extracted with phenol/chloroform and precipitated with EtOH in the presence of 20 μg of carrier RNA. The pellet was resuspended in 100 μl of Tris-EDTA and mixed with 12.4 ml of CsCl (d = 1.75 g/ml). The mixture was centrifuged in a Ti 70.1 rotor for 45 h at 30,000 rpm. After this, fractions were collected and counted in a liquid scintillation counter. The density of each fraction was determined by refractive index.

Production of Fusion Proteins

Glutathione-S-transferase (GST)–cyclin A and His-Cip fusion proteins were made and purified as described in Hua et al. (1997) and Guadagno et al. (1996), respectively. The XORC2 and His-cdc6 expression vector was provided by Dr. W. Dunphy (California Institute of Technology, Pasadena, CA) (Carpenter et al., 1996; Coleman et al., 1996). XORC2 was overexpressed in bacterial strain BL21Lys(S). Gel-purified XORC2 protein was injected into rabbits to produce anti-ORC2 antibody. Crude serum was used for Western blotting and immunodepletion. R48-ubiquitin and WT-ubiquitin expression plasmid was provided by Dr. J. Callis (Univeristy of California, Davis, CA).

A fragment of *Xenopus* cdc6 was cloned by PCR amplification with degenerate primers designed using the similar sequence shared by Cdc18 and Cdc6 from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae.* (Gavin et al., 1995). The 5′ primer was 5′-GGATCCCCHG-GNACHGGNAARAC-3′ and the 3′ primer was 5′-CTCGAGTC-CATYTCRTCVARNAC-3′. The PCR reaction (100 μl) was carried out using 20 ng *Xenopus* oocyte cDNA as template. Primers, dNTPs, *Taq* polymerase, and buffer conditions were chosen as recommended by the manufacturer. The reaction mix was first heated at 94°C for 2.5 min. Then it was cycled 36 times at 94°C for 1 min; 55°C for 2 min; and 72°C for 2 min. An extra 5 min was added to the last 72°C extension step. The reaction was run on a 1.0% agarose gel, and a 300-bp fragment was isolated, subcloned, and then sequenced. The sequence was identical to a part of the Xcdc6 GenBank sequence (these data are available from GenBank/EMBL/DDBJ under accession number U66558). Cdc6 fragment was then overexpressed as a GST fusion protein (Guadagno and Newport, 1996). The protein was gel purified and injected into rabbit for antibody production. The serum was purified through GST-coupled, CNBr-activated beads (Pharmacia Biotechnology Inc., Piscataway, NJ) and the flow through was collected for Western blotting.

Immunodepletion and Western Blotting

Immunodepletion of ORC was carried out as described by Walter and Newport, 1997. Briefly, for depletion of 100 μl of interphase extract, 20 μl of crude serum, and 20 μl packed volume protein A–Sepharose beads (Pharmacia Biotechnology, Inc.) were incubated at room temperature for 30 min. After this, the beads were washed and incubated with crude interphase extract for 45 min. The extract was then separated from the beads by low speed centrifugation. This procedure was repeated twice to deplete >95% of the ORC from the extract. Western blotting was carried out as described (Harlow and Lane, 1988).

Sperm and Nucleus Pelleting Experiments

To pellet the sperm, 10 μl of sample was diluted 10-fold with egg lysis buffer (ELB) (Fang and Newport, 1991), layered onto a 17% sucrose cushion made in the same buffer, and then spun at 4°C in a microfuge fitted with a horizontal rotor for 20 s. The supernatant was removed, and the pellet was resuspended in SDS–sample buffer, and then processed for Western blotting.

To assay chromatin-associated proteins inside nuclei, nuclei were generated in vitro in the presence of aphidicolin. 20 μl of sample was layered onto a 15% sucrose cushion made in ELB (Fang and Newport, 1991), and then centrifuged for 20 s at 14,000 rpm at 4°C. The pellet was resuspended in 100 μl of chromatin extraction buffer (see above), and layered onto a 17% sucrose cushion made in the same buffer. The sample was spun again and the pellet was recovered and resuspended in SDS–sample buffer and subjected to Western analysis. This method was used routinely for nuclei extraction (high stringency wash). "Low stringency wash" was carried out as described by Kubota et al. (1995). Briefly, the sample containing nuclei was diluted 10-fold with extraction buffer (100 mM KCl, 2.5 mM $MgCl_2$, and 50 mM Hepes-KOH, pH 7.5) containing 0.25% Triton X-100. The diluted sample was layered on top of 30% sucrose in EB, and then centrifuged at 4°C for 15 min. The pellet was resuspended in SDS–sample buffer and used as chromatin fraction (Kubota et al., 1995).

Miscellaneous Procedures

Interphase cytosol and membrane fractions were made as described previously (Fang and Newport, 1991). DNA replication was assayed by incorporation of radioactive dATP (Kornbluth et al., 1992). The intensity of the bands was quantitated by phosphoimager (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

Addition of Cyclin A to Egg Extracts Selectively Displaces ORC from Chromatin

High speed centrifugation of crude *Xenopus* egg extracts conveniently separates the membranous components of the extract from the cytosolic components (Newport, 1987). The cytosolic fraction contains the ORC and MCM complexes as well as cdc6 (Chong et al., 1995, Madine et al., 1995a, Carpenter et al., 1996; Coleman et al., 1996). When sperm chromatin is added to this cytosolic fraction, ORC, cdc6, and MCM assemble onto the chromatin within 20 min (Hua, X.H., unpublished observation). However, in the absence of membrane components nuclei cannot assemble, and initiation of DNA replication at preassembled origin sites does not occur (Newport, 1987; Sheehan et al., 1988). We have observed that if the concentration of active cdk2–cyclin E kinase is increased in the cytosolic fraction before sperm chromatin addition, the kinase prevents MCM proteins from associating with the chromatin but does not block binding of the ORC (Hua et al., 1997). As expected, if membrane components are added to such an extract, the chromatin assembles into nuclei but fails to initiate DNA replication. By contrast, if chromatin is added to the cytosol first, ORC and MCM proteins form preinitia-

tion complexes, and these complexes are not released when cdk2–cyclin E kinase is added later. When membranes are added to this cytosol, nuclei form around the chromatin and a single round of DNA replication occurs (Hua et al., 1997).
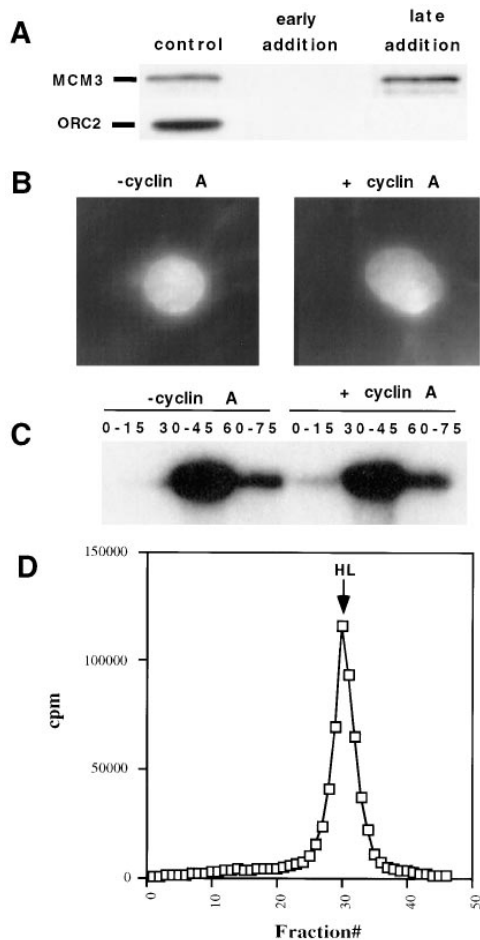
To examine whether changes in the binding of ORC and MCM proteins occur when cdc2–cyclin A concentration is varied, a similar set of experiments were conducted. We observed that when cyclin A was added to the cytosolic fraction it rapidly associated with free cdc2 to form active cdc2–cyclin A kinase (Hua et al., 1997; data not shown). When sperm chromatin was added to cytosol pretreated with moderate amounts of cyclin A, both ORC and MCM proteins failed to associate with chromatin based on Western blots of sperm chromatin recovered from the cytosol (Fig. 1 A, early addition). Thus, unlike cdk2–cyclin E, in which addition of the kinase before chromatin blocks MCM but not ORC binding, early addition of cdc2–cyclin A kinase prevents both ORC and MCM from binding to chromatin. A trivial explanation for this difference might be that addition of these levels of cyclin A induces mitosis. That this is not the case is indicated by microscopic observations of the chromatin, which showed that the DNA remained decondensed and did not form the condensed chromosomes typically observed upon entry into mitosis (data not shown).

To examine whether ORC and MCM proteins that are already associated with chromatin, can be displaced by moderate levels of cdc2–cyclin A kinase activity, the following was done. Sperm chromatin was incubated in egg cytosol for 30 min to allow ORC and MCM proteins to associate with chromatin (Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995a; Carpenter et al., 1996). Recombinant cyclin A was then added. After a further 30 min, the sperm chromatin was separated from the cytosol by brief centrifugation, and associated ORC and MCM proteins were determined by Western blotting using anti-ORC2 and -MCM3 antibodies. Surprisingly, MCM remained associated, but all of the ORC was released (Fig. 1 A, late addition). Therefore, increased concentrations of cdc2–cyclin A kinase selectively promote the release of ORC from replication complexes without causing MCM to be displaced.

Chromatin Lacking ORC Replicates Normally

In both yeast and metazoan cells, ORCs remain associated with origins throughout S phase of the cell cycle (Diffley et al., 1994; Coleman et al., 1996). Although it has been shown that binding of ORC to chromatin is a prerequisite for assembly of MCM proteins into preinitiation complexes, it is not known if, once this has occurred, ORC must remain bound at origins to carry out other functions required for initiation of DNA replication. Our observation that addition of cyclin A to preformed ORC–MCM–chromatin complexes selectively displaces ORC from chro-

Figure 1. Cyclin A dissociates prebound ORC protein from chromatin, whereas DNA replication is not affected by this treatment. (*A*) Western blots of sperm-associated MCM3 and ORC2 with "early" and "late" addition of cyclin A. In "early addition" experiments, cytosol was preincubated with 100 nM of cyclin A for 30 min. After this, sperm chromatin was added to 5,000/µl. After a further 30-min incubation, the reaction was diluted fivefold in ELB (see experimental procedures), and then centrifuged through a 15% sucrose cushion. Chromatin-bound MCM3 and ORC2 were assayed by Western blotting using anti-MCM3 and anti-ORC2 antibodies. In "late addition" experiments, cytosol was preincubated with 5,000/µl of sperm for 30 min before 100 nM of cyclin A was added. The reaction was incubated for anther 30 min. The sample was processed in the same way as described above. Chromatin-bound MCM3 and ORC2 without addition of cyclin A is shown in *control*. Early addition of cyclin A prevents both MCM and ORC from binding to chromatin, whereas late addition of cyclin A dissociates prebound ORC but not MCM from chromatin. (*B*) Sperm was incubated with cytosol for 30 min. After this the sample was split into two parts, into which either membrane (−*cyclin A*), or membrane and 100 nM of cyclin A (+*cyclin A*) was added. After another 60-min incubation, nuclei were fixed and stained with Hoechst. Pictures show that nuclei form normally in presence of cyclin A. (*C*) DNA replication with late addition of cyclin A. Cytosol was preincubated with sperm chromatin (2,000/µl) for 30 min. After this, the sample was split into two parts and 100 nM of cyclin A was added to one (+*cyclin A*). After a further 30-min incubation, membrane was added to both parts and DNA replication was assayed by pulse labeling at 0–15, 30–45, 60–75-min time intervals. Incorporation of radioactive dATP occurred normally with late addition of cyclin A. (*D*) Density substitution assay with late addition of cyclin A. Replication was carried out as in *B*, +*cyclin A* reaction, except with 0.5 mM bromodeoxyuridine and 0.5 mM MgCl₂. After 2 h, the DNA was isolated and analyzed by CsCl density centrifugation. The expected location of heavy-light DNA is indicated (HL: $d = 1.74$ g/ml).

matin before initiation, allows us to address whether ORC binding is required for initiation events after MCM binding. To do this, ORC–MCM–chromatin complexes were formed in egg cytosol. After this, cyclin A was added to the cytosol to displace ORC, and then membranes were added. After addition of membranes, visual observation of chromatin by fluorescent microscopy showed that nuclei formed around the chromatin (Fig. 1 *B*), confirming that addition of this concentration of cyclin A does not induce a mitotic state. Importantly, when replication of the ORC-depleted chromatin within these nuclei was monitored by measuring the amount of radioactively labeled dCTP incorporated into DNA, we observed that both the kinetic rate and absolute amount of replication was identical between cyclin A–treated and control (no cyclin A added) samples (Fig. 1 *C*). That the observed replication was due to semi-conservative synthesis and represented a single round of synthesis was confirmed by adding Br-dUTP to the extract. The results of this experiment showed that the end product of replication was a single-labeled band of density equal to one strand of normal-density DNA annealed to a heavy-density, newly synthesized strand containing Br-dUTP (Fig. 1 *D*).

After Cyclin A–dependent Release, ORC Does Not Reassociate with Chromatin in Nuclei

The experiments above strongly suggest that once ORC has acted to load MCM onto chromatin, it may not be required for the late steps in initiation. However, this conclusion is based on the displacement of ORC from chromatin by cyclin A before the incorporation of the chromatin into nuclei. If conditions within nuclei differed from those in the cytosol, it is possible that in the presence of cyclin A, ORC could reassociate with chromatin after nuclear assembly. To examine this possibility, we incubated sperm chromatin in cytosol for 30 min, added cyclin A to displace ORC, and then added membranes to allow nuclei to form around the chromatin. This reaction mixture was then split in half. Half was used to measure DNA replication, which was normal compared to a non–cyclin A–treated control (Fig. 2 *B*). The other half was treated with aphidicolin to block postinitiation DNA synthesis, thereby preventing displacement of MCM during further incubation. After a 60-min incubation, during which nuclei formed and grew, the nuclei were pelleted through a sucrose cushion, permeabilized with NP-40 to release non–chromatin-bound ORC, and then washed again. Chromatin-associated ORC and MCM were then assayed from Western blots (Fig. 2 *A*). These experiments showed that whereas ORC remained associated with chromatin in control extracts (not treated with cyclin A; Fig. 2 *A*, *control*), it was not found associated with chromatin isolated from nuclei assembled in the presence of cyclin A (Fig. 2 *A* early and late addition). The small amount of ORC observed in these nuclear pellets was also observed in samples lacking DNA, and is most likely because of a small amount of ORC nonspecifically associated with membranes (data not shown). By contrast, the amount of MCM associated with control and late cyclin A–treated nuclei remained the same. Together these results demonstrate that after formation of nuclei in cyclin A–treated extracts, ORC does not reassociate with the
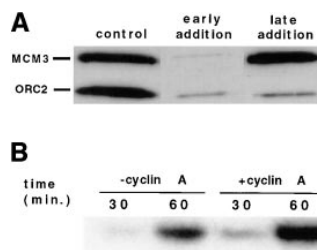

Figure 2. After being displaced from chromatin by cyclin A, ORC does not reassociate with chromatin after forming nuclei. The reaction was carried out as in Fig. 1 *A* except membrane was added at the end of the reaction. The samples were incubated for a further 60 min to assay chromatin-bound MCM3, ORC2, and DNA replication. (*A*) Nuclei were pelleted through a sucrose cushion, permeabilized, and then spun again through a second cushion (see Materials and Methods). Pellet fractions were recovered and chromatin-bound MCM and ORC determined by Western analysis. After forming nuclei, ORC remains displaced from chromatin. The small amount of ORC observed with early and late addition are likely to be the ORC nonspecifically associated with membrane. To prevent MCM dissociation from chromatin as a result of replication, aphidicolin (50 μg/ml) was added in these reactions. (*B*) DNA replication assay. The incorporation of radioactive-labeled dATP is identical between control and late cyclin A–treated extract.

chromatin. Again, these data strongly support the conclusion that once MCM is bound to DNA, continued association of ORC at origins is not required for late steps leading to the initiation of DNA replication.

ORC-deficient Chromatin Replicates Normally in ORC-depleted Extracts

It has been shown that *Xenopus* egg extracts depleted of ORC are unable to replicate added chromatin (Coleman et al., 1996). The results presented above strongly predict that ORC–MCM–chromatin complexes treated with cyclin A to completely release ORC should, after isolation, replicate when added back to an ORC-depleted egg extract. To test this, chromatin was incubated in cytosol and then treated with cyclin A to release ORC (Fig. 3 *A*, *late addition*). This ORC-depleted chromatin was then isolated away from the cytosol by centrifugation. Similarly, chromatin was isolated from extracts that were treated with cyclin A before addition of sperm (Fig. 3 *A*, *early addition*). These manipulations result in the purification of two types of chromatin, one type lacking ORC but containing MCM (MCM+ chromatin) and one type lacking both ORC and MCM (MCM− chromatin). MCM− chromatin was first used to test the efficiency of ORC depletion by mixing in either mock-depleted or ORC-depleted extract. With mock-depleted, normal extract, ORC and MCM proteins assembled into preinitiation complexes at origins and DNA replicated normally (Fig. 3 *C*, *1*). However, in ORC-depleted extract, DNA replication was significantly inhibited (Fig. 3 *C*, *2*) because of the almost-complete absence of ORC proteins (96% depleted; Fig. 3 *B*). We next examined how MCM+ chromatin, when mixed with ORC-depleted extract, would replicate. Our result shows clearly that the ORC-free chromatin replicated efficiently in an extract that lacks ORC proteins (Fig. 3 *C*, *3*). These results demonstrate that preinitiation complexes formed on chromatin are fully functional without ORC proteins, and MCM is the key component for later initiation events. This result,
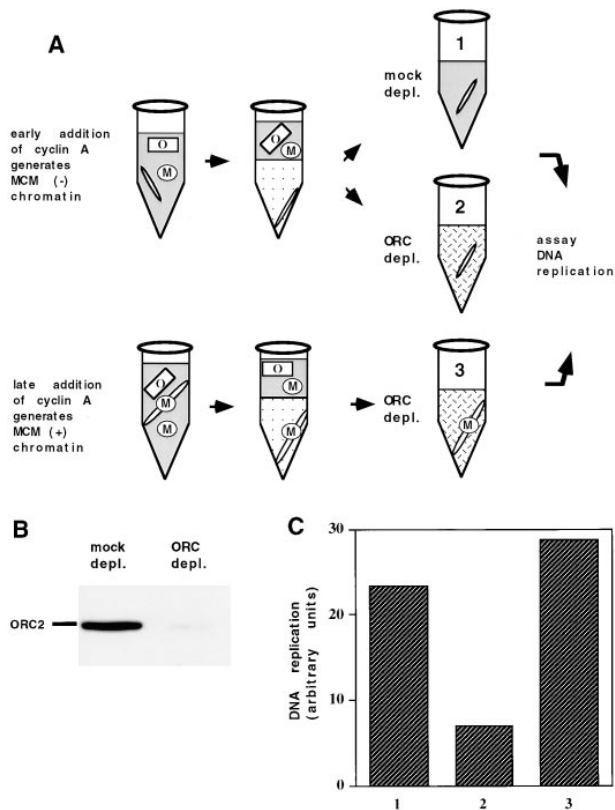
Figure 3. MCM-associated, ORC(−) chromatin can replicate in an ORC-depleted extract. (*A*) Diagram of the experiment in (*C*). Early and late addition of cyclin A produces two types of sperm. When cyclin A is added before sperm (*early addition*), neither MCM (*M*) nor ORC (*O*) can bind to chromatin. This treatment results in sperm lacking MCM and ORC (*top*). When cyclin A is added after sperm (*late addition*), only ORC dissociates from chromatin. This treatment produces sperm containing MCM but lacking ORC (*bottom*). These two types of sperm were isolated by centrifugation though a sucrose cushion. After removing the supernatant and sucrose cushion, sperm pellets were resuspended in either mock-depleted (*1*) or ORC-depleted extract (*2* and *3*), and DNA replication was assayed. (*B*) Western blot showing the depletion of ORC2 from the extract. Anti-ORC antibodies were coupled to protein A–Sepharose beads and incubated with interphase extract for 45 min. The beads were removed by low speed centrifugation. After three cycles of depletion, >96% of the ORC was removed. Preimmune serum was used as control (*mock depl.*). (*C*) Replication assays described in *A* were carried out for 90 min, and incorporation of radioactive dATP was quantitated. MCM (+) chromatin replicates efficiently in ORC-depleted extract (*3*). MCM (−) chromatin replicates well only in mock-depleted (*1*) but not in ORC-depleted extract (*2*).

in combination with those described above, confirms that the ORC is not essential for late initiation steps.

The cdc6 in Preinitiation Complexes Is Rapidly Removed from Chromatin After MCM Loading

Binding of the MCM protein complex to chromatin is dependent on the preassociation of both the ORC and cdc6 with chromatin (Coleman and Dunphy, 1996; Rowles et al., 1996). Given the results presented above it was important to determine whether the association of cdc6 with chroma-
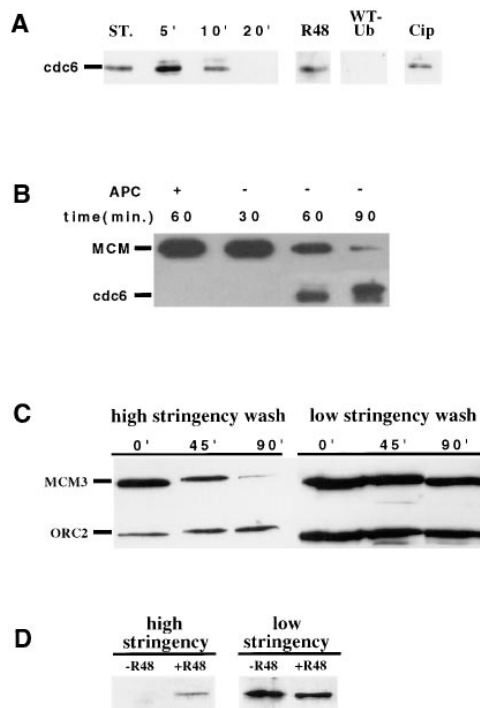

Figure 4. Chromatin-bound cdc6 is rapidly degraded and does not reassociate with DNA until after initiation of replication. (*A*) 2,000/µl of sperm chromatin was added into interphase extract and incubated for 5, 10, and 20 min. Sperm was then pelleted and associated cdc6 was analyzed by Western blot (chromatin-bound cdc6; *5′*, *10′*, and *20′*). Cdc6 associates with chromatin at 5 min, and completely disappears from chromatin after 20 min. Alternatively, interphase cytosol was preincubated with HisUbR48 (*R48*), His-Ub (*WT-Ub*), or His-Cip (*Cip*) for 20 min, and then sperm chromatin was added and incubated for a further 30 min. Chromatin-associated cdc6 is stabilized by UbR48 and Cip but not by WT-Ub. His-cdc6 is shown as a standard (*ST.*). (*B*) Interphase extract was incubated with sperm chromatin and membrane with or without aphidicolin. After the indicated period of time, the reactions were stopped by diluting fivefold with ELB and pelleting through a sucrose cushion. The nuclei were permeabilized and pelleted again. Chromatin-associated cdc6 and MCM were analyzed by Western blotting using specific antibodies. (*C*) Sperm and membrane were added into interphase cytosol and DNA replication was allowed to occur for the indicated time periods (*t* = 0 represents aphidicolin arrest). For each time point, the sample was divided into two parts, and different extraction methods were used to determine chromatin-associated MCM3 and ORC2 (see Materials and Methods). Dissociation of MCM3 from chromatin during DNA replication can be seen only with the *high stringency wash.* (*D*) Interphase cytosol was incubated alone (−*R48*) or with R48 (+*R48*) for 20 min. Sperm, membrane, and aphidicolin (50 µg/ml) were then added to both and the reactions incubated for a further 60 min. After this, both reactions were split into two parts. Nuclei were extracted using high stringency condition for one part and low stringency condition for the other. Chromatin-associated cdc6 was then assayed by Western blot. With a high stringency wash, cdc6 cannot be detected on chromatin in absence of R48. In the low stringency wash of the same nuclei, significant amounts of cdc6 can be seen attached to chromatin both with and without R48.

tin after MCM binding was essential for late steps in the initiation process or whether after MCM loading, cdc6, like ORC, was not required for the late steps leading to initiation. To pursue this issue an anti–*Xenopus* cdc6 antibody was used to follow the association of cdc6 with chromatin. Specifically, sperm chromatin was incubated in egg cytosol for different periods of time, and then isolated and analyzed for cdc6 content from Western blots using the anticdc6 antibody as probe. The results from this experiment demonstrated that cdc6 associated with sperm chromatin within 5 min after addition to the cytosol (Fig. 4 *A*). However, this association was transient, decaying rapidly and little, if any, cdc6 remained bound to the chromatin by 20 min.

With respect to how cdc6 might be removed from chromatin, several experiments in yeast have demonstrated that cdc6 and its homologue, cdc18, are unstable proteins that are degraded rapidly (Nishitani and Nurse, 1995; Piatti et al., 1995; Muzi-Facolni et al., 1996). The degradation of cdc18 is mediated by a ubiquitin-dependent proteolytic system (Kominami and Toda, 1997). To test whether this is the case in egg extracts, we used a mutation in the ubiquitin protein R48, which competitively blocks poly-ubiquitination, thereby inhibiting proteolysis by the ubiquitin pathway (Chau et al., 1989). We found that when R48-ubiquitin (final concentration = 30 μM) was added to cytosol, cdc6 remained stably associated with sperm chromatin for at least 30 min (Fig. 4 *A*, *R48*). The same amount of wild-type His-ubiquitin had no effect (Fig. 4 *A*, *WT-Ub*). This suggests the possibility that after its initial rapid association with chromatin, cdc6 is normally degraded by a ubiquitin-dependent pathway. Because cdk2 has been implicated as playing a role in controlling cdc6 turnover in yeast (Jallepalli and Kelly, 1996), we investigated whether displacement of chromatin-associated cdc6 in egg extracts could be blocked by addition of the cdk2 inhibitor Cip. The results from this experiment (Fig. 4 *A*, *Cip*) showed that, like R48, addition of Cip (final concentration = 1 μM) to egg cytosol stabilized the association of cdc6 with chromatin for at least 30 min. Together these experiments demonstrate that cdc6 rapidly associates with chromatin added to egg cytosol and this association facilitates loading of MCM protein. However, once this process is completed cdc6 is removed from the chromatin, possibly by a cdk2 kinase–activated, ubiquitin-dependent proteolytic system.

During the course of DNA replication, MCM proteins are irreversibly displaced from chromatin (Chong et al., 1995; Madine et al., 1995*b*; Todorov et al., 1995; Coue et al., 1996; Kubota et al., 1996; Hua et al., 1997). Because the results described above suggest that removal of cdc6 from origin complexes may depend on the presence of MCM, we determined whether free cdc6 present in extracts could stably reassociate with chromatin after the replication-dependent displacement of MCM complexes from chromatin. To do this, sperm was incubated in cytosol for 30 min. After this, membranes were added and DNA replication was allowed to proceed. At different times during replication, the amount of cdc6 and MCM associated with chromatin was determined from Western blots. No cdc6 was detected during initiation and early stages of replication (Fig. 4 *B*, −*APC*, *30 min*). However, as replication proceeded and MCM was displaced form the chromatin, we observed that

cdc6 reassociated with chromatin (Fig. 4 *B*, −*APC*, *60 min*). After replication and complete release of MCM, significant rebinding of cdc6 occurred (Fig. 4 *B*, −*APC*, *90 min*). Importantly, this newly bound cdc6 was now stably associated even in the absence of R48-ubiquitin addition. Together these results suggest that if, as the R48 data suggests, cdc6 is removed from preinitiation complexes by degradation, and then such degradation may only occur after MCM has been loaded onto chromatin. This direct association of cdc6 and MCM at preinitiation complexes may form a hybrid site that allows factors to bind and either release or degrade cdc6. After replication, the absence of MCM on DNA would allow free cdc6 to rebind stably to chromatin. Our observations are consistent with recent data showing that overexpression of cdc6 at G2 in yeast causes cdc6 to reassociate with chromatin (Tanaka et al., 1997).

Our results are at odds with data that cdc6 remains associated with chromatin until DNA replication initiates (Coleman et al., 1996). These conclusions were largely based on Western and immunofluorescence studies of when cdc6 is bound to chromatin. However, we have observed that some replication proteins can bind nonspecifically to chromatin, depending on the buffer conditions used during washing of the chromatin for Western analysis. Because of this we compared our washing procedures with that of Coleman et al. (1996). Using our chromatin washing conditions we found that, as expected (Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995*b*; Todorov et al., 1995; Coue et al., 1996) MCM3 protein is displaced from chromatin as replication proceeds to completion (Fig. 4 *C*, *high stringency wash*). By contrast, and as observed by Coleman et al., (1996) very little MCM3 appears to be released from chromatin during replication when this chromatin is isolated using less stringent procedures (Fig. 4 *C*, *low stringency*). Similarly, using our washing procedure we find little, if any, cdc6 associated with chromatin inside nuclei, except when R48-ubiquitin is included in the extract from the beginning (Fig. 4 *D*, *high stringency*). By contrast, using the less-stringent methods of Coleman et al. (1996), cdc6 is found associated with nuclei both in the absence and presence of R48 (Fig. 4 *D*, *low stringency*). One trivial explanation of these results is that high stringency washing denatures replication proteins and strips them off chromatin. However, this seems unlikely based on the observation that at early time points, MCM and cdc6 stably associate with chromatin under the high stringency washing condition (Fig. 4, *A* and *C*). In addition, after replication, cdc6 is observed to again be bound to chromatin with a high stringency wash. Based on these observations, we believe that it is possible that the characterization of cdc6 within nuclei reported previously may represent cdc6 that is bound to chromatin nonspecifically. In particular, cdc6 may show a high degree of nonspecific binding to chromatin that is covered with MCM complex.

Removal of cdc6 from Chromatin Correlates with Initiation of DNA Replication

Recently, ubiquitin-mediated proteolysis has been implicated in initiation of DNA replication (Yew and Kirschner, 1997). To determine whether displacement of cdc6 might
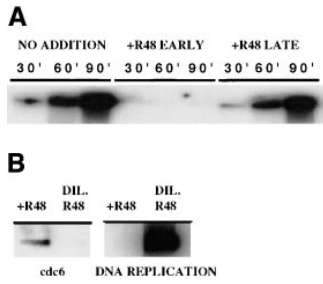
Figure 5. Inhibition of protein degradation prevents DNA replication. (*A*) Interphase cytosol was incubated with either R48 (*+R48 early*) or with sperm chromatin (*+R48 late*) for 20 min. Then sperm chromatin or R48 was added, respectively, and the samples were incubated for another 20 min. After this, membrane was added to both, and DNA replication at indicated time points was assayed. DNA replication with early addition of R48 is strongly inhibited, whereas replication with late addition is identical to the control (*no addition*). (*B*) The effect of R48 can be reversed by dilution. 20 µl of interphase cytosol was incubated with R48 for 20 min. Sperm was then added and incubated for another 30 min. After this the reaction was split into two halves and 90 µl of fresh cytosol was added into only one (*Dil. R48*). The samples were then assayed for both cdc6 content and DNA replication. 10-fold dilution of R48 destabilizes chromatin-bound cdc6 and restores DNA replication.

be essential for allowing a late initiation step to occur, we tested whether DNA replication was inhibited by R48-ubiquitin addition and whether this inhibition was restricted to the short temporal period during which cdc6 was associated with chromatin. Specifically, 30 µM of R48-ubiquitin was added to cytosol 20 min before or after sperm chromatin addition. Membranes and radioactively labeled dATP were added to each sample 30 min after the addition of R48, and then DNA replication was assayed by incorporation of label into DNA. The results from this experiment (Fig. 5 *A*) showed that addition of R48-ubiquitin to extracts after the transient interaction of cdc6 with chromatin had no effect on DNA replication (Fig. 5 *A*, compare *no addition* with *+R48 late*). By contrast, when R48-ubiquitin was added to the extract 20 min before chromatin, it both stabilized the association of cdc6 with chromatin (see above) and completely inhibited DNA replication (Fig. 5 *A*, *+R48 early*). This result suggests that displacement of chromatin-bound cdc6, possibly by degradation, may be essential for the proper execution of late initiation steps. To pursue this possibility further, R48-ubiquitin, sperm chromatin, and membranes were added to an extract and the extract was incubated for 60 min until nuclei had formed around the chromatin. These nuclei failed to replicate their DNA (Fig. 5 *B*, *DNA replication*, *+R48*). Moreover, when these nuclei were isolated by centrifugation, and then permeabilized with NP-40 to release free cdc6, the remaining chromatin contained cdc6 (Fig. 5 *B*, *cdc6*, *+R48*). After nuclear formation, DNA replication could be rescued by addition of nine volumes of untreated extract to reduce the R48 concentration (Fig. 5 *B*, *DNA replication*, *Dil. R48*). After such a dilution, cdc6 was absent on nuclear chromatin (Fig. 5 *B*, *cdc6*, *Dil. R48*). The results of these experiments show that the inhibition of DNA replication by R48-ubiquitin is reversible and that removal, likely by degradation of chromatin-bound cdc6, is strongly correlated with such rescue. Thus, cdc6 is a potential candidate whose proteolysis is required for initiation of DNA replication.

Cdk2 Kinase Functions in Initiation After ORC

Together our results suggest that neither ORC nor cdc6 proteins may be required for late initiation steps. This conclusion is founded on the assumption that DNA replication cannot initiate before nuclear formation. The data supporting this assumption are derived from experiments showing that when chromatin is added to egg cytosol lacking membrane components, little, if any, radioactive nucleotide is incorporated into DNA (Newport, 1987; Sheehan et al., 1988). Also, attempts to observe short, radioactively labeled nascent replication products on denaturing gels have failed to demonstrate that such products exist (Walter, J., and J. Newport, unpublished data). However, similar results could be obtained if initiation occurred in the cytosol, but further chain elongation was strongly inhibited. A more rigorous means of showing that initiation cannot occur in the cytosol would be to demonstrate that at least one initiation step cannot occur until nuclei have formed around chromatin. In particular, cdk2 kinase is known to be essential for initiation of DNA replication, but not required for elongation (Strausfeld et al., 1994). As such, we determined when cdk2 activity was required for replication, and whether there is a cdk2-dependent step after both ORC and cdc6 have been displaced from chromatin.

To determine if cdk2 is required for establishing ORC–MCM–chromatin complexes, 1 µM of cdk2 kinase inhibitor Cip was added to cytosol to completely block kinase activity. After 20 min, chromatin was added to the cytosol. After a further 30-min incubation the chromatin was isolated by centrifugation and the amount of ORC and MCM bound was determined by Western blotting. The result of this experiment showed that the total amount of ORC and MCM proteins bound to chromatin was independent of cdk2 kinase activity (Fig. 6 *A*). Therefore, as expected, the ORC-dependent loading of MCM proteins onto chromatin in egg cytosol does not appear to require cdk2 kinase activity.

To determine if cdk2 kinase activity was required for initiation of replication at a step after completion of ORC function and removal of cdc6 from preinitiation complexes, the following was done. Sperm chromatin was added to egg cytosol and incubated for 30 min to allow ORC and MCM to bind and cdc6 to be cleared from chromatin (Fig. 6 *B*). After this, cyclin A was added to the cytosol to displace ORC. The MCM–chromatin complexes generated were then isolated by centrifugation. This ORC-cdc6–deficient chromatin was divided in half and added either to an ORC-depleted membrane–cytosol extract or the same extract containing 1 µM Cip. DNA replication in each of these extracts was then determined (Fig. 6 *C*). As expected, the ORC-cdc6–depleted chromatin replicated at normal rates in ORC-depleted extract. By contrast, when this same chromatin was added to extracts containing Cip, little if any replication was observed (compare Fig. 6 *C*, −*Cip* and +*Cip*). Thus, after removal of ORC and cdc6 proteins from chromatin, initiation of DNA replication is still dependent on cdk2 kinase. This experiment demonstrates two points. First, it shows that in egg extracts, initiation of DNA replication does not occur before nuclear envelope formation. If it had occurred, replication in this experiment would be insensitive to Cip. Second, it demonstrates
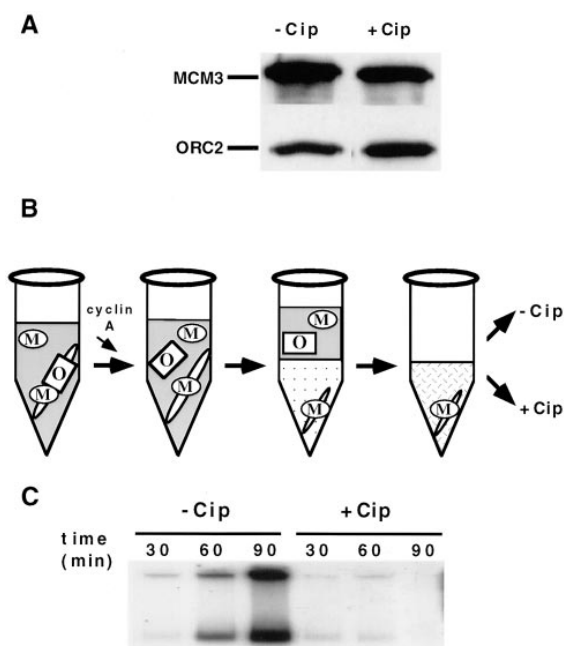
Figure 6. After MCM binds to chromatin, DNA replication is still inhibited by Cip. (*A*) Western blot showing the effect of Cip on MCM3 and ORC2 binding. Interphase cytosol was incubated either with buffer (−*Cip*) or with Cip (+*Cip*) for 20 min. Sperm (5,000/µl) was added and incubated for a further 30 min. The samples were then diluted and pelleted through a sucrose cushion. Pellet fractions were recovered and subject to Western blotting analysis using specific antibodies. The result shows that the binding of ORC and MCM to chromatin is insensitive to Cip addition. (*B*) An illustration of the DNA replication assay performed in *C.* Interphase cytosol was preincubated with sperm (5,000/µl) for 30 minutes (step 1). 100 nM of cyclin A was then added and incubated a further 30 min (step 2). This treatment removes prebound ORC (*O*) without displacing MCM (*M*). Sperm chromatin was then isolated from the cytosolic mixture by centrifugation through a sucrose cushion (step 3). This sperm chromatin was then resuspended in ORC-depleted extract (step 4). The sample was then divided into two parts and Cip was added to only one of them. DNA replication was then assayed. (*C*) DNA replication assay showing incorporation of radioactive dATP at 30, 60, and 90 min after adding ORC-depleted extract. Under these experimental conditions DNA replication is greatly inhibited by Cip.

that there is a cdk2-dependent step during the initiation process that is independent of both ORC and cdc6 proteins.

Discussion

In this study we have presented evidence strongly supporting the conclusion that the ORC completes its function before the final step(s) involved in initiation of DNA replication. In support of this conclusion, we have used moderate levels of cdc2–cyclin A kinase as a tool to displace ORC from preinitiation complexes (Fig. 1 *A*). We find that when these ORC-depleted initiation complexes are assembled into nuclei, they function efficiently to initiate a single round of semi-conservative DNA replication (Fig. 1, *B–D*). To ensure that the replication observed under these condi-

tions is truly independent of ORC activity, we have also shown that isolated ORC-depleted, preinitiation complexes replicate normally when added back to an extract lacking ORC (Fig. 3 *C*). Together these results suggest that after the ORC-dependent loading of cdc6 and MCM proteins onto chromatin (Coleman et al., 1996), ORC function is not essential for the final steps required for origin activation. Thus, the major function of the ORC may be to select origin sequences and load proteins required for initiation at these sites. With respect to steps downstream of ORC and cdc6 function, we have shown that at least one of these late steps requires active cdk2–cyclin E kinase. In support of this conclusion, we have shown that isolated ORC-cdc6–depleted chromatin initiation complexes fail to replicate when added to ORC-deficient extracts containing the cdk2 kinase inhibitor Cip (Fig. 6 *C*). This result demonstrates, that after the completion of ORC and cdc6 functions, there is at least one cdk2 kinase–dependent step remaining before initiation can occur.

A number of excellent studies demonstrate that the events leading to initiation of DNA replication are divided into two stages during the cell cycle (Diffley et al., 1994; Piatti et al., 1996; for reviews see Diffley, 1996; Wuarin and Nurse, 1996). Specifically, during the first stage, which occurs during early G1, cdk2 kinase activity is low and ORC, cdc6, and MCM assemble onto chromatin to form potential preinitiation complexes (Yan et al., 1993, Zwerschke et al., 1994; Chong et al., 1995; Cocker, 1995; Kubota et al., 1995; Madine et al., 1995; Piatti et al., 1996) (Fig. 7). The assembly of these initiation complexes occurs sequentially (Coleman et al., 1996). ORC binds first and is required for cdc6 binding; and cdc6 assembly is required for MCM binding (Fig. 7 *A*). During late G1-S, the activation of cdk2 kinase(s) inhibits assembly of further preinitiation complexes until completion of mitosis (Dahmann et al., 1995; Hua et al., 1997). Thus, the formation of preinitiation complexes appears to occur during early G1, whereas the final steps leading to the activation of these complexes occur during late G1-S. Our results raise the possibility that ORC function may be limited to the early G1 steps, during which preinitiation complexes form, and may not participate in the late G1 steps occurring after activation of cdk2 kinase.

Displacement of ORC from Chromatin in Metazoans during Mitosis

In yeast ORC remains associated with chromatin throughout the entire cell cycle including mitosis (Diffley et al., 1994). By contrast, in metazoan organisms such as *Xenopus*, ORC dissociates from the condensed chromatin during mitosis (Coleman et al., 1996). The different binding properties of ORC to chromatin during mitosis in yeast and metazoan cells could be because of differences in either chromatin structure or ORC modification in these different cell types. For example, chromosome condensation in yeast during mitosis is rather limited compared to condensation in metazoans. As such, the tight condensation of chromatin in metazoans might passively displace ORC from chromatin at mitosis, whereas the partial condensation occurring in yeast might leave ORC bound. Alternatively, in metazoan cells ORC may be modified such
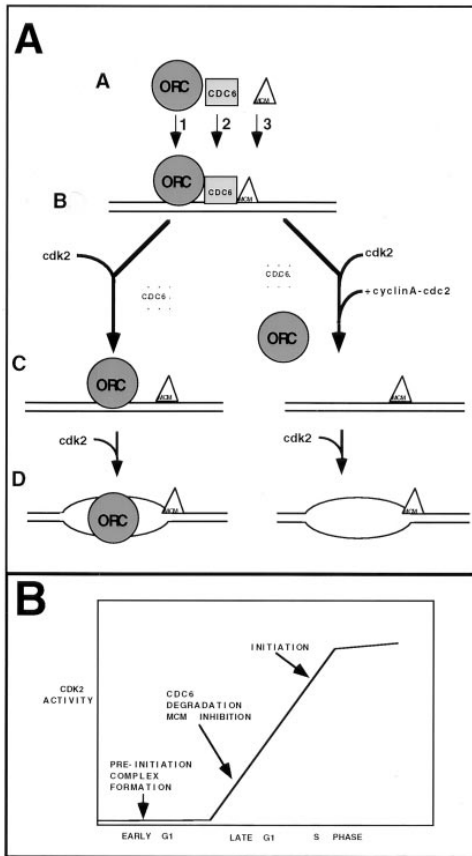
Figure 7. Steps leading to initiation of replication. During early G1, cdk2 kinase activity is absent and ORC, cdc6, and MCM proteins bind to chromatin in a sequentially dependent manner to generate preinitiation complexes. During late G1, high levels of cdk2 activity accumulate. This activity leads to the degradation of cdc6. We have shown that activation of moderate levels of cdc2–cyclin A kinase at this time leads to displacement of ORC, creating an initiation complex containing only MCM. After cdc6 is displaced from preinitiation sites there is a second cdk2-dependent step that must occur before actual initiation takes place.

that its affinity for chromatin is significantly reduced at mitosis. Our observations argue that at least this second possibility is occurring. Specifically, when cyclin A is added to extracts at the moderate levels used in our studies, it associates with endogenous cdc2 to generate active kinase. At these levels of cdc2–cyclin A kinase, ORC cannot associate with chromatin, and chromatin-bound ORC dissociates from chromatin (Fig. 1 *A*). In both of these cases the chromatin appears decondensed and forms normal nuclei, and in the latter case, DNA replication occurs normally. This suggests that in the presence of this concentration of cdc2–cyclin A, ORC is modified such that its affinity for chromatin is greatly reduced. Whether this modification is directly because of cdc2–cyclin A kinase or to an activity regulated by cdc2 kinase has yet to be determined. During the normal cell cycle, cdc2–cyclin A activity increases rapidly just before cells enter mitosis. Based on the observation that moderate levels of cyclin A displace ORC from chromatin (this report), whereas higher concentrations drive extracts into mitosis (Roy et al., 1991; Strausfeld et al., 1996; Hua, X.H., unpublished observation), it is likely that dis-

placement of ORC by cyclin A kinase is part of an early preconditioning process that normally occurs just before cells enter mitosis. By stably arresting an extract in this transitional state we have generated a unique opportunity to investigate initiation events that are independent of the ORC.

The Role of cdc6 during Initiation Complex Formation

With respect to the role cdc6 plays in initiation of replication, our results strongly suggest that cdc6 acts transiently to load MCM protein onto chromatin, and then is removed from the chromatin, possibly by a cdk2 ubiquitin-dependent proteolytic degradation; and cdc6 is not required for late initiation steps. In support of this conclusion we have shown that cdc6 transiently associates with chromatin during the time interval required to load MCM onto chromatin. After this, cdc6 is absent on chromatin (Fig. 4 *A*).

Our results also suggest that in metazoans, degradation of cdc6 during late G1 may be an essential component in potentiating origins for initiation later during S phase. In support of this we find that if R48-ubiquitin is added to stabilize chromatin-bound cdc6, DNA replication is strongly inhibited (Fig. 5). By contrast, if R48 is added to extracts after cdc6 has been removed from chromatin, DNA replication occurs normally. Further, we have observed that reversal of the R48 inhibition of DNA replication (by dilution) is accompanied by removal of cdc6 from chromatin. This strict correlation between association of cdc6 with chromatin and inhibition of DNA replication strongly suggests that cdc6 must be displaced, likely by degradation, before an initiation complex can become competent for the final steps of initiation. Importantly, because this displacement step occurs before actual initiation, it suggests that cdc6 function, like ORC function, may primarily be involved in the loading of MCM proteins onto origin sites. Our results are fully consistent with a recent report demonstrating that a dominant-negative form of the ubiquitin-conjugating protein, cdc34, blocks DNA replication when added to *Xenopus* extracts (Yew and Kirschner, 1997), and suggests that cdc6 may be a target of the cdc34 protein.

We have shown that cdc6 is rapidly removed from chromatin after MCM is loaded. This suggests that dissociation or degradation of chromatin-bound cdc6 is dependent on the association of MCM with DNA. In support of this we find that after the replication-dependent displacement of MCM from chromatin, cdc6 protein again associates stably with chromatin presumably at origin sites. These observations suggest that during preinitiation complex formation, cdc6 is essential for loading MCM and that this same loading event generates a complex that results in the displacement or degradation of cdc6 from the prereplication complex. Interestingly, we have also observed that once cdc6 is displaced from preinitiation sites, addition of R48 does not allow free cdc6 to stably reassociate with the site until replication has initiated and MCM is displaced (Hua, X.H., unpublished observations). This suggests that after cdc6 displacement, MCM and ORC occlude a cdc6-binding site in the prereplication complex. Such a process could serve, in part, to ensure that origins fire only once per S phase. However, because cdc6 can reassociate with origins after the replication-dependent displacement of MCM (Tanaka

et al., 1997), it appears that the process preventing MCM from reassociating with chromatin after replication is largely responsible for restricting origin firing to a single round per cell cycle.

cdk2 Kinase Functions Twice during Initiation

With respect to the role cdk2 kinase plays during the initiation process, our results indicate that cdk2 is not required for the assembly of preinitiation complexes. When Cip is present in an extract, ORC, cdc6, and MCM still assemble into preinitiation complexes (Fig. 6 *A*). However, as discussed above, cdk2 is essential for the displacement of cdc6 in preinitiation complexes and this displacement removes a barrier to initiation. Importantly, our results strongly suggest that cdk2 participates in a second step of the initiation process, and that this step is independent of both ORC and cdc6 proteins. Specifically, under conditions in which both the ORC and cdc6 have been removed from chromatin, DNA replication is still sensitive to the cdk2 inhibitor Cip (Fig. 6 *C*). Potential targets for this second cdk2 step include phosphorylation of MCM proteins by cdk2 (Schulte et al., 1995; Coue et al., 1996; Hendrickson, et al., 1996), or the cdk2-dependent activation of other proteins essential for initiation such as cdc7 (Hereford and Hartwell, 1974; Yoon et al., 1993; Dowell et al., 1994), or a yet to be identified helicase.

The identification of a second cdk2-dependent step may have important implications for the temporal regulation of replication events during the cell cycle. In early G1 the absence of cdk2–cyclin E activity allows preinitiation complexes, consisting of ORC, cdc6, and MCM, to form. In late G1 the activation of small amounts of cdk2-cyclin may serve to both prevent MCM from associating with DNA (Hua et al., 1997), and degrading cdc6, thereby blocking the formation of new initiation complexes and potentiating existing complexes for initiation. If the second cdk2-dependent initiation step can only occur at high cdk2 levels, this requirement would serve to impose a temporal lag between these two events and the initiation of DNA replication, which demarks the actual entry into S phase. Such a lag would ensure that these processes occurred before initiation, thus eliminating the possibility that an origin might be used twice during S phase (Fig. 7 *B*).

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