A Role for Cdk2 Kinase in Negatively Regulating DNA Replication during S Phase of the Cell Cycle

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Abstract. Using cell-free extracts made from *Xenopus* eggs, we show that cdk2-cyclin E and A kinases play an important role in negatively regulating DNA replication. Specifically, we demonstrate that the cdk2 kinase concentration surrounding chromatin in extracts increases 200-fold once the chromatin is assembled into nuclei. Further, we find that if the cdk2–cyclin E or A concentration in egg cytosol is increased 16-fold before the addition of sperm chromatin, the chromatin fails to initiate DNA replication once assembled into nuclei. This demonstrates that cdk2–cyclin E or A can negatively regulate DNA replication. With respect to how this negative regulation occurs, we show that high levels of cdk2–cyclin E do not block the association of the protein complex ORC with sperm chromatin but do prevent association of MCM3, a protein essential for replication. Importantly, we find that MCM3 that is prebound to chromatin does not dissociate when cdk2–cyclin E levels are increased. Taken together our results strongly suggest that during the embryonic cell cycle, the low concentrations of cdk2–cyclin E present in the cytosol after mitosis and before nuclear formation allow proteins essential for potentiating DNA replication to bind to chromatin, and that the high concentration of cdk2–cyclin E within nuclei prevents MCM from reassociating with chromatin after replication. This situation could serve, in part, to limit DNA replication to a single round per cell cycle.

Genetic and biochemical observations have partially characterized the events which potentiate DNA for replication during S phase of the eukaryotic cell cycle. In yeast, the multiprotein complex ORC binds to specific DNA sequences, and this association is essential for converting these sites into origins used during DNA replication (Bell et al., 1993; Rao et al., 1995; Donovan and Diffley, 1996). Although metazoan origin sequences have not yet been rigorously defined, it is likely that the metazoan homologues of the yeast ORC proteins serve a similar function (Gavin et al., 1995; Carpenter et al., 1996). Recent biochemical experiments using cell-free extracts derived from *Xenopus* eggs have demonstrated that both cdc6 and the MCM family of proteins only associate with chromatin after the metazoan ORC complex has bound to the chromatin (Coleman et al., 1996). Furthermore, this study showed that the association of MCM with chromatin was dependent on the prebinding of the cdc6 protein. Cdc6 is a 61-kD protein that appears to be essential for generating active origins (Bueno and Russell, 1992; Kelly et al., 1993; Liang et al., 1995; Nishitani and Nurse, 1995; Piatti et al., 1995; Coleman et al., 1996), and the MCM proteins form a multisubunit complex that is both essential for DNA replication (Hennessy et al., 1991; Yan et al., 1991, 1993; Dalton and Whitebread, 1995) and likely involved in limiting replication to a single round per cell cycle (Tye, 1994; Kubota et al., 1995; Chong et al., 1995; Madine et al., 1995*a*,*b*).

A number of studies have shown that activation of DNA replication at S phase is dependent on cdk2 kinase activity. For example, *Xenopus* extracts which normally replicate exogenously added chromatin templates efficiently, fail to do so after removal of cdk2 kinase activity (Fang and Newport, 1991; Jackson et al., 1995). Precisely how cdk2 contributes to the activation of replication at the molecular level is currently unknown. Interestingly, a number of excellent experiments demonstrate that cdk activity also functions to limit replication to a single round each cell cycle. For example, using the fission yeast *Schizosaccharomyces Pombe* as a model system, it was found that cells containing certain temperature sensitive mutations in either the cdc2 protein or cyclin B initiate a second round of DNA replication without first entering mitosis (Brock et al., 1991; Hayles et al., 1994; Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995). Similarly, *Drosophila* embryonic cells lacking cyclin A undergo endoreduplication (Sauer et al., 1995). It has also been shown that when synchronized rat fibroblasts are treated with inhibitors known to block cdc2 activity, multiple rounds of replication occur in the absence of mitosis (Usui et al., 1991). Together these results argue strongly that during G_2 of the cell cycle cdk kinase activity is essential for blocking endoreduplication,

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and that this mechanism is evolutionarily conserved between all eukaryotic cell types.

More recently, it has been proposed that the absence of cdk kinase activity at the end of mitosis generates a permissive period during which proteins essential for potentiating DNA for replication, such as cdc6 and MCM, can associate with DNA and that the activation of cdk during late G_1 inhibits further association (Dahmann et al., 1995, Piatti et al., 1996). Based on this model the periodic oscillation of cdk activity could allow potentiation to occur during early G_1 and inhibit subsequent potentiation at all other times during the cell cycle. Although the model is both appealing and consistent with the cyclic oscillation of cdc2 activity during the somatic cell cycle, it is not easily reconciled with the conditions found during the early embryonic cell cycles of many species. In particular, during the first 12 cell cycles of *Xenopus* embryogenesis, DNA replication is limited to a single round per cell cycle, yet, cdk2–cyclin E activity is constant during the entire cell cycle (Fang and Newport, 1991; Rempel et al., 1995; Howe and Newport, 1996). Clearly, based on the model described, the presence of cdk2 throughout the embryonic cell cycle should inhibit associations between chromatin and proteins involved in replication, thereby blocking DNA replication. In considering this inconsistency it has been proposed that in eggs the distribution of cdk2 between different cellular compartments might provide a means for reconciling the apparent conflict between the somatic and embryonic situation (Su et al., 1995). Specifically, if cdk2 is actively transported into nuclei, the actual concentration of kinase surrounding chromatin in the cytoplasm during mitosis might be significantly lower than the concentration of kinase surrounding this same chromatin during S phase when it is contained within nuclei. As such, the cell cycle–dependent compartmentalization of cdk2 could generate a gradient of cdk2 activity which would allow essential replication proteins to bind to chromatin at the end of mitosis immediately before nuclei form and prevent further binding after nuclei form.

To test this possibility we have increased the amount of cdk2–cyclin E activity surrounding chromatin in *Xenopus* extracts before the formation of nuclei. Our results demonstrate that under these conditions the ORC complex binds to chromatin normally. However, MCM3 fails to associate, and after nuclear formation, DNA replication does not initiate. Overall, our results strongly suggest that the cell cycle–dependent compartmentalization of active cdk2 kinase within nuclei participates in regulating DNA replication during the cell cycle.

Materials and Methods

Preparation of Fractionated Interphase Cytosol and Membrane

The egg extracts used in these studies were prepared by fractionating crude interphase egg extracts (Fang and Newport, 1991) in a centrifuge (TL100; Beckman Instruments, Inc., Fullerton, CA) at 55,000 rpm for 90 min. The cytosol was collected and rapidly frozen in liquid nitrogen. The membrane fraction was diluted in 1.5 ml of egg lysis buffer (ELB; Fang and Newport, 1991) containing 10 μg/ml of aprotinin and leupeptin. Following this it was layered on top of a 0.5 ml sucrose cushion (8.7% in ELB) and spun at 20,000 rpm for 20 min. The membrane was aliquoted, rapidly frozen in liquid nitrogen, and stored at −80°C.

Isolation of Fusion Proteins

pGEX-KG containing *Xenopus* cyclin A was transformed into TOPP 3 *Escherichia coli* bacteria strain (Strategene, La Jolla, CA). The construction and transformation of pGST–cyclin E and pGST-cdk2 has been described previously (Guadagno and Newport, 1996). Recombinant proteins were expressed and purified from soluble fractions by affinity chromatography on glutathione–sepharose beads as described (Solomon et al., 1990). Fractions that contain recombinant protein were concentrated and buffer exchanged as previously described (Guadagno and Newport, 1996).

P13 Precipitation and Western Blot

P13 was coupled to CNBr-activated Sepharose beads as described in Dunphy et al. (1988). Packed P13 beads (20 μl) were incubated with 50 μl of diluted supernatant or resuspended pellet fractions at 4°C for 1 h. The P13 beads were then pelleted, washed extensively with wash buffer (Fang and Newport, 1991), and eluted with SDS-PAGE sample buffer. The samples were fractionated on 10% SDS-PAGE gels and then analyzed by standard Western blot techniques (Harlow and Lane, 1988) using appropriate antibodies and ECL reagents (Amersham Corp., Chicago, IL).

Fractionation Experiments

In the cyclin E transport assay, 10 μl of extract was incubated with 2 μl of membrane fraction and various concentrations of demembraned sperm for 60 min. After this the samples were layered on a 15% sucrose cushion in a capillary tube and spun in a microfuge fitted with a horizontal rotor for 10 min at 4°C. Cyclin–cdk complexes in both the supernatant and the pellet fractions were precipitated with p13 beads and subjected to Western blot analysis.

To pellet the sperm, 10 μl of sample was diluted fivefold with ELB, layered onto a 17% sucrose cushion, and centrifuged for 10 min at 14,000 rpm at 4°C. The supernatant and pellet fractions were then mixed with SDS sample buffer. One fifth of the supernatant and all of the pellet fraction were loaded on 10% SDS-PAGE gel and processed for Western blotting.

DNA Replication Assay

DNA replication was assayed by incorporation of [γ-^{32}P]dATP. Pulse or continuous labeling was carried out as described by Kornbluth et al. (1992). DNA replication was also assayed by incorporation of biotin-labeled dUTP. 1 μl of Bio-dUTP was added to 200 μl of extract at the beginning of the reaction. After a 1-h incubation, 20-μl aliquots were taken and chilled on ice for 10 min. The samples were then diluted and fixed with 800 μl of ELB with 2 mM EGS for 20 min at room temperature. After this the samples were layered on PBS containing 25% sucrose and spun onto polylysine-treated coverslips. The coverslips coated with nuclei were washed, fixed with 3.7% formaldehyde in PBS for 10 min, and then washed another three times. Nonspecific binding was blocked by incubating with PBS containing 10% FCS and 0.1% Triton X-100 for 10 min. The coverslips were then rinsed and incubated for 1 h with PBS containing 10% FCS and 1:100 dilution of streptavidine-conjugated Texas red. After this, the coverslips were rinsed and mounted with 25% glycerol with 0.01% Hoechst.

Immunofluorescence Microscopy

Immunofluorescence staining of MCM3 protein on sperms was performed as described in Yan and Newport (1995). The fixed sperms were first stained with rabbit anti-MCM3 antibody (1:1,000 in PBS) for 1 h, washed, and then stained with goat–anti rabbit rhodamine conjugate (Sigma Chemical Co., St. Louis, MO 1:100 in PBS). The samples were mounted with Hoechst solution (0.01% in ELB) and viewed with a fluorescence microscope.

Results

When sperm chromatin is added to *Xenopus* egg extracts prepared in the presence of the protein synthesis inhibitor

cycloheximide, the chromatin is first encapsulated into nuclei and then efficiently replicated once (Lohka and Masui, 1984; Blow and Laskey, 1986; Newport, 1987). The absence of further rounds of replication demonstrates that the mechanisms which limit replication to a single round per cell cycle are active in extracts. Moreover, because such extracts lack cyclins A, B1, and B2, cdc2 kinase is inactive. This demonstrates that unlike yeast cells, inhibition of multiple rounds of DNA replication in egg extracts does not require mitotic cdc2 kinase activity. However, these extracts do contain cdk2–cyclin E kinase activity (Fang and Newport, 1991). Because cdk2–cyclin E normally accumulates within nuclei, it is possible that the concentrations of cdk2 surrounding chromatin in the cytosol and the nucleus differ and that this difference might participate in regulating DNA replication.

To pursue this possibility we have determined whether the ckd2–cyclin E concentration in nuclei is higher than that present in the cytosol. To do this, different numbers of sperm chromatin were added to a fixed volume of egg extract. After a 60-min incubation, during which the chromatin was both assembled into nuclei and replicated, the nuclei were separated from the surrounding cytosol by sedimentation through a 15% sucrose cushion. The resulting nuclear pellet was then assessed for cdk2–cyclin E content by Western blot analysis using both anti-cdk2 and anti–cyclin E antibodies. The results of these experiments (Fig. 1, *A* and *B*) demonstrated that the amount of cdk2–cyclin E complex present in the nuclear pellet increased linearly up to 2,000 sperm/μl. No further increase in cdk2–cyclin E was observed at concentrations above 2,000 sperm/μl, indicating that all of the endogenous cdk2–cyclin E complex initially present in the cytosol had been transported into the newly formed nuclei. This conclusion is supported by direct comparison of the cdk2–cyclin E in both the cytosol and nuclear fractions in an extract containing 2,000 nuclei/μl (Fig. 1 *C*). It is clear that under these conditions the vast majority (>95%) of the cyclin E is nuclear, and relatively little (<5%) remains in the cytosol. Based on the observation that nuclear cdk2–cyclin E is readily released from interphase nuclei treated with mild detergent (0.1% Triton X-100), the majority of the cdk2–cyclin E transported into nuclei does not appear to be tightly associated with chromatin (results not shown).

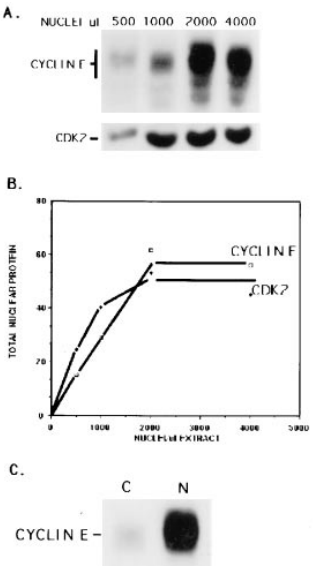

Figure 1. Compartmentalization of cdk2–cyclin E within nuclei assembled in egg extracts. (*A*) Different numbers of demembranated sperm were added to reconstituted interphase extracts containing both cytosol and membrane. After a 60-min incubation to allow nuclear assembly and transport to occur, the reconstituted nuclei were separated from the cytosol by centrifugation through a 15% sucrose cushion. These nuclear fractions were assayed for both cdk2 and cyclin E by Western blotting, using anti-cdk2 and anti-cyclin E antibodies as probes. (*B*) The intensity of the bands in *A* were quantitated by densitomitry. *Open squares*, cyclin E; *closed squares*, cdk2. (*C*) Interphase extract was incubated for 60 min with 2,000 sperm/μl and membrane. Cytosolic (*C*) and nuclear (*N*) fractions were separated by centrifugation through a 15% sucrose cushion. All of the cdk2 and cyclin E in both fractions was then bound to P13-Sepharose beads, washed, eluted with SDS-PAGE sample buffer, and analyzed by Western blotting with anti–cyclin E antibody. Under these "high sperm" conditions almost all of the cyclin E initially present in the cytosol had been transported into nuclei.

This experiment allows us to calculate the difference in cdk2–cyclin E concentration present initially in the cytosol, before nuclei form, relative to the concentration of cdk2–cyclin E inside nuclei. To do this, the average diameter of nuclei formed in the extract was measured from microscopic observations using beads of defined size as a reference standard. This nuclear diameter (17 ± 2 μm) was used to calculate nuclear volume. Based on these calculations, 2,000 nuclei occupy a volume of 5×10^{-3} μl. Because all of the cdk2–cyclin E initially present in 1 μl of cytosol is transported into 2,000 nuclei, these results demonstrate that the cdk2–cyclin E activity initially present in the cytosol is concentrated 200-fold after transport into nuclei. Previous studies have estimated that the cdk2–cyclin E concentration in egg cytosol is 0.06 μM (van Renterghem et al., 1994), whereas our results demonstrate that the concentration of cdk2–cyclin E within nuclei is 12.0 μM. Therefore, before the assembly of a nuclear membrane around sperm chromatin, mitotic chromosomes, or the DNA in permeablized nuclei, the chromatin will be surrounded by levels of cdk2–cyclin E kinase activity 200-fold lower than will surround it once the chromatin has acquired a nuclear envelope.

High Cytoplasmic cdk2–cyclin E Levels Block DNA Replication

Although the level of cdk2–cyclin E activity does not vary significantly during the embryonic cell cycle (Fang and Newport, 1991; Rempel et al., 1995; Howe and Newport, 1996), the experiments presented above demonstrate that the actual concentration of kinase surrounding chromatin will vary over 200-fold depending on whether the cell cycle is at the end of mitosis (no nucleus) or S phase (intact nucleus). To investigate whether this gradient of kinase activity can participate in regulating DNA replication, *E. coli*–produced recombinant *Xenopus* cdk2 and cyclin E proteins were added to extracts to increase the cytosolic pool of cdk2–cyclin E surrounding chromatin before nuclear assembly. Specifically, cdk2–cyclin E was added to purified egg cytosol to a final concentration of 1 μM, or 16 times the normal cytosolic concentration. This extract was incubated for 30 min, and then membranes and sperm chromatin (1,000/μl) were added to the extract. Based on microscopic observations, the added chromatin and membrane assembled into normal nuclei, and the DNA decondensed (Fig. 2 *A*). In control extracts lacking exogenous cdk2–cyclin E, pulse labeling with radioactive dATP demon-

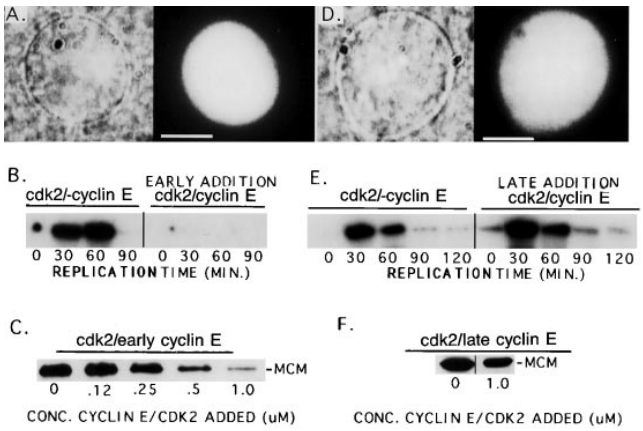

Figure 2. High concentrations of cdk2–cyclin E inhibits both DNA replication and the binding of MCM3 to chromatin. (*A*) Purified egg cytosol containing 1 μM of added cdk2–cyclin E was preincubated for 30 min. After this incubation, membrane and sperm (1,000/μl) were added to the extract. As shown, by using phase optics (*left*), these sperm formed intact nuclei, and by fluorescent staining (*right*), the DNA decondensed. (*B*) Interphase cytosol was preincubated alone (− cdk2–cyclin E), or with 1 μM of cdk2–cyclin E (*cdk2–Cyclin E*) for 30 min. After this membrane and 1,000 sperm/μl were added. The autoradiogram shows ^{32}P incorporated into DNA during 15 min pulses starting at indicated times after sperm addition. In the absence of cyclin E, replication occurred normally, while in the presence of cyclin E it was strongly inhibited at all time points. (*C*) cdk2–cyclin E was added to interphase cytosol to the final concentrations indicated. After a 30-min incubation, 1,000 sperm/μl were added to each reaction, the reactions were incubated for another 30 min, and then the sperm were pelleted. The pellets were resuspended in SDS-PAGE sample buffer. MCM3 bound to the sperm chromatin was determined by Western blotting using anti-MCM3 antibody. As shown, MCM3 binding to chromatin was inhibited as the concentration of cdk2–cyclin E increased from 0.12 to 1.0 μM. (*D*) Interphase cytosol was first incubated with 1,000 sperm/μl for 30 min. 1 μM of cdk2–cyclin E was then added together with membrane. Photographs show a typical nucleus that formed under these conditions. (*E*) Interphase cytosol was first incubated with 1,000 sperm/μl for 30 min. After this, membrane was added with or without 1 μM of cdk2–cyclin E and DNA replication was assayed as in *B*. Replication in both extracts was identical, demonstrating that late addition of cdk2–cyclin E does not inhibit chromatin from replicating. (*F*) Sperm was added to cytosol to a final concentration of 1,000 sperm/μl. After a 30-min incubation, the indicated amounts of cdk2–cyclin E were added, and the reaction was incubated for another 30 min. At the end of the incubation the sperm were pelleted and assayed for MCM3 by Western blotting as described in *C*. As shown, late addition of cdk2–cyclin E does not inhibit binding of MCM3 to chromatin. Bars: (*A* and *D*) 10 μm.

strated that replication initiated, as usual, after a 20–30 min lag (Newport, 1987) continued for 30 min and then stopped (Fig. 2 *B*, − cdk2–cyclin E). By contrast, extracts which were preincubated with cdk2–cyclin E 30 min before addition of sperm and membranes failed to show any significant DNA replication (Fig. 2 *B*, *Early Addition*). This experiment demonstrates that if the concentration of cdk2–cyclin E present in the cytosol during nuclear assembly is 16-fold higher than normal, replication is strongly inhibited.

To determine whether chromatin which is preincubated in normal extract becomes refractive to the inhibition of replication by high cdk2–cyclin concentrations, sperm chromatin (1,000 μl) was preincubated in purified egg cytosol (i.e., lacking membranes) for 30 min. After this the extract was split into two aliquots. To one aliquot, membranes alone were added, while to the other, both cyclin E–cdk2 (final concentration 1 μM) and membranes were added. Again, nuclei assembled normally in both samples, and the encapsulated DNA decondensed (Fig. 2 *D*). Importantly, under these conditions, DNA replication was identical in both samples (Fig. 2 *E*). This experiment demonstrates that addition of 1 μM cdk2–cyclin E, by itself, does not inhibit DNA replication. Rather, the time of addition of the kinase relative to the addition of sperm chromatin determines whether replication is inhibited. Replication is blocked if cdk2–cyclin E is present in the cytosol at high concentrations before chromatin addition. However, replication occurs normally if chromatin is exposed to cytosol before the addition of cdk2–cyclin E.

High Cytosolic Concentrations of cdk2–cyclin E Blocks Binding of MCM3 to Chromatin

The results above suggest that high concentrations of cytosolic cdk2–cyclin E may inhibit proteins required for DNA replication from associating with chromatin. To examine this possibility, we investigated how the association of MCM3 with chromatin is affected by cytosolic cdk2–cyclin E concentration. Specifically, different concentrations of cdk2–cyclin E protein were added to pure cytosol and incubated for 30 min, and then sperm chromatin was added to the cytosol. After another 30 min incubation the sperm chromatin was separated from the cytosol by centrifugation through a 17% sucrose cushion, and MCM3 binding to chromatin was analyzed on Western blots using anti-*Xenopus* MCM3 antibody as a probe. The results from these experiments showed quite clearly that the association of MCM3 with chromatin decreased as cdk2–cyclin E concentration increased (Fig. 2 *C*). Little decrease was observed at cdk2–cyclin E concentrations twofold higher than endogenous levels (0.12 μM), a 50% reduction was observed at fourfold higher levels (0.25 μM), an 80% reduction occurred at eightfold higher levels (0.5 μM), and a 90% reduction occurred at 16-fold higher than endogenous levels (1 μM). By contrast, when sperm chromatin was incubated first for 30 min in pure egg cytosol and then 1 μM cdk2–cyclin E was added to the extract, little decrease in the binding of MCM3 to chromatin was observed (Fig. 2 *F*). Thus, for at least one essential DNA replication protein, MCM3, high concentrations of cdk2–cyclin E blocks binding of the protein to chromatin. Importantly, these results also show that once MCM3 is bound to chromatin, increasing the concentration of cdk2–cyclin E, as would occur following nuclear formation, does not cause dissociation of the protein from chromatin.

With respect to how cdk2–cyclin E blocks the association of MCM with chromatin, it has been shown that the ORC complex binds to DNA before MCM and that this interaction is a prerequisite for MCM binding to chromatin (Coleman et al., 1996). Therefore, if high concentrations of cdk2–cyclin E modified ORC so as to prevent it from interacting with DNA, this modification would also

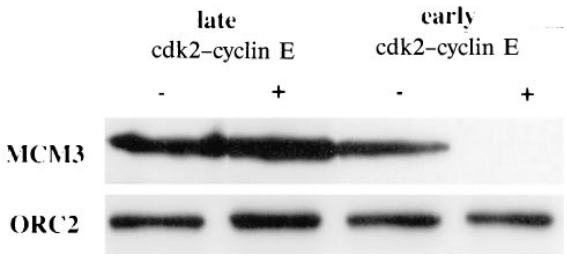

Figure 3. High concentration of cdk2–cyclin E doesn't inhibit ORC2 from binding to chromatin. 500 nM of cdk2–cyclin E was added either before (*early cdk2–cyclin E*) or 30 min after (*late cdk2–cyclin E*) sperm addition. Chromatin fractions were extracted with ELB containing 0.1% NP-40 and spun through a sucrose cushion containing 0.1% NP-40. The pellet fractions were analyzed for MCM3 and ORC2 content by Western blotting with specific antibodies. MCM3 binding to chromatin is inhibited by early addition of cdk2–cyclin E, while ORC2 binding is unaffected.

prevent MCM from associating with chromatin. To test this possibility we used an anti-ORC2 antibody to examine whether binding of the ORC complex to chromatin was sensitive to cdk2–cyclin E concentration. The results of this experiment showed quite clearly that concentrations of exogenously added cdk2–cyclin E which completely inhibited MCM binding had no effect on the association of ORC with DNA (Fig. 3). Therefore, the inhibitory effects of cdk2-cyclin E on the interaction of MCM with chromatin appear to involve a step downstream of the association of ORC with DNA.

Inhibition of DNA Replication Requires cdk2–cyclin E Activity

In principle, increasing the cdk2–cyclin E concentration in the cytosol could inhibit DNA replication by two distinct mechanisms. The increased kinase activity of cdk2 could lead to the phosphorylation and inhibition of components required for assembly of MCM onto DNA. Alternatively, the increased concentration of cdk2–cyclin E protein complex could bind to and sequester activities required for mediating the association of MCM with chromatin (Piatti et al., 1996). Two experiments were performed to distinguish between these kinase- and complex-dependent possibilities. In one experiment a high concentration of cdk2–cyclin E was added to an extract, and the extract was then incubated for 30 min. After this the cdk2 kinase inhibitor Cip was added to the extract to inactivate cdk2–cyclin E kinase activity. Sperm chromatin was then added and assayed for MCM binding as described above. The result of this experiment (Fig. 4 *A*) demonstrated that the inactivation of cdk2–cyclin E kinase activity by Cip restores binding of MCM to chromatin. This result strongly suggests that cdk2 kinase activity itself, rather than the sequestration of factors by cdk2–cyclin E complex, is responsible for blocking the association of MCM with chromatin.

If cdk2–cyclin E inhibited the association of MCM with chromatin by sequestering activities needed for mediating this association, then addition of a kinase inactive cdk2–cyclin E complex should also inhibit this association. To test this possibility, different concentrations of a kinase defective cdk2, cdk2K33R, and cyclin E were added to extracts.

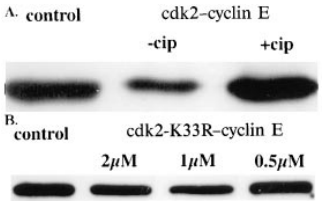

Figure 4. Inhibition of MCM3 binding requires active cdk2–cyclin E kinase activity. (*A*) 500 nM of cdk2–cyclin E was first incubated with interphase cytosol for 30 min (*cdk2–cyclin E*). The reaction was then split into two parts, and cip (final 600 nM) was added to one half (+ cip). After a 15-min incubation, sperm was added (final 5,000/μl) to both, and the reactions were carried out for another 30 min. Chromatin-associated MCM3 was analyzed by Western blotting using anti-MCM3 antibody. Control shows the amount of MCM3 bound to chromatin without cdk2–cyclin E treatment. When the kinase activity of cdk2–cyclin E was blocked by cip, it could no longer inhibit MCM3 binding. (*B*) Interphase cytosol was incubated either alone (*control*) or with indicated concentrations of cdk2-K33R–cyclin E for 30 min. After this incubation, sperm chromatin was added to 5,000/μl. After being incubated for another 30 min, the sperm was pelleted. MCM3 bound to the sperm chromatin was assayed as in *A*. cdk2-K33R–cyclin E, which is a kinase inactive complex, does not inhibit MCM3 from binding to chromatin.

After a 30 min incubation, sperm chromatin was added to these extracts, and after a further 30 min incubation, the association of MCM with this chomatin was determined (Fig. 4 *B*). The results from this experiment quite clearly showed that the kinase inactive cdk2–cyclin E complex did not inhibit MCM from binding to chromatin. This result in combination with the experiment above demonstrates that it is the catalytic activity of cdk2, not the amount of cdk2–cyclin E complex, which prevents MCM from associating with chromatin.

Generation of Pseudo G_1 and G_2 Nuclei in the Same Extract

The results presented above show that chromatin incubated in cytosol containing high concentrations of cdk2–cyclin E fails to replicate when it assembles into nuclei. Further, our results show that cdk2–cyclin E is concentrated within nuclei as a result of nuclear transport. This sequence of events makes two predictions. First, it predicts that chromatin assembled into nuclei in extracts initially containing high concentrations of cdk2–cyclin E will not replicate if the cytosol is diluted. This will occur because the cdk2–cyclin E is already compartmentalized within nuclei and no longer subject to dilution. The second prediction is that fresh nuclei added to this diluted cytosol would replicate. This would occur because the new chromatin would be assembled into nuclei in cytosol containing low concentrations of cdk2–cyclin E.

To test these predictions, cdk2–cyclin E was added to purified cytosol and incubated for 30 min. After this, sperm chromatin (final 1,000/μl) and membranes were added. Aliquots of this sample were removed, incubated with radioactively labeled dATP, and then assayed for DNA replication. As expected, nuclei assembled in the presence of high cdk2–cyclin E failed to initiate replication during the subsequent 60 min incubation (Fig. 5 *A*, *left*). To test the effects of dilution on this inhibition, extracts were diluted with 4 vol of untreated extract. This addition dilutes cdk2–

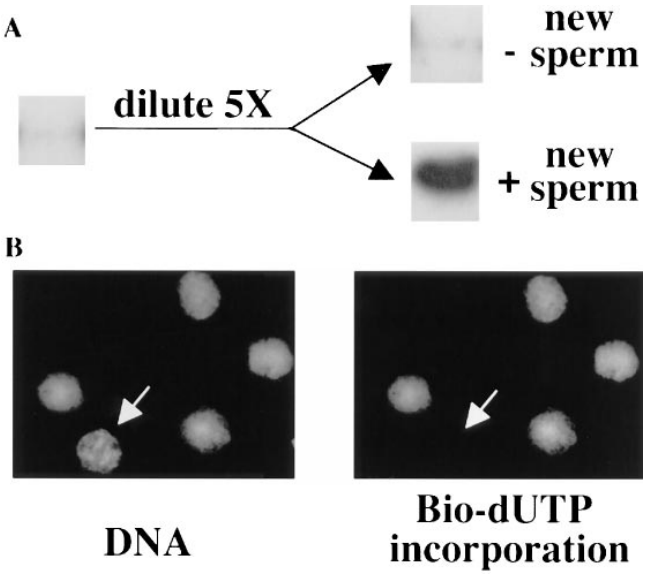

Figure 5. Nuclei assembled in the presence of high cdk2–cyclin E fail to initiate DNA replication when the cytosol is diluted. (*A*) Interphase cytosol was first incubated with 1 μM of cdk2–cyclin E for 30 min. After this preincubation, sperm chromatin (1,000 sperm/μl) and membrane were added. Aliquots were removed and incubated with [^{32}P]dATP to determine early replication (*left*). As expected, high cdk2–cyclin E concentration blocked replication. The remainder of the mixture was incubated for a further 60 min and then diluted with 4 vol of fresh extract containing both cytosol and membrane but lacking cdk2–cyclin E. The diluted reaction was then divided in half, and new sperm chromatin (1,000/μl) was added to one half. Radioactively labeled dATP was then added to both reactions, and DNA replication was assayed after a further 60-min incubation. Nuclei assembled in the presence of high cdk2–cyclin E concentrations failed to replicate after dilution of the extract (− new sperm) while new nuclei added to such a diluted extract replicated normally (+ new sperm). (*B*) After dilution, an aliquot was taken from the sample "+ new sperm," and bio-dUTP was added. After 1 h of incubation, the nuclei were spun onto a coverslip, stained with straptavidine-conjugated Texas red, and mounted with Hoechst. Five nuclei were visualized in this field (*left*), and four of them had bio-dUTP incorporated (*right*).

cyclin E remaining in the cytosol but should not dilute cdk2–cyclin E that has been compartmentalized within nuclei. The result of this experiment showed that nuclei preassembled in extracts initially containing high cdk2–cyclin E concentrations, failed to initiate DNA replication upon dilution (Fig. 5 *A*, *right*, *top*, − new sperm). This observation is consistent with the prediction that once cdk2–cyclin E is compartmentalized in nuclei, dilution of cdk2–cyclin E remaining in the cytosol will not restore replication.

Importantly, we find that chromatin that is added to the extracts after dilution replicates normally (Fig. 5 *A*, *right*, *bottom*, + new sperm). To show that under these conditions replication was exclusive to the chromatin added after dilution, biotin-dUTP was added to the extract. After this, the extract was incubated for 30 min, and then the nuclei were fixed and stained with streptavidin conjugated to the fluorophor Texas red. Nuclei which had replicated (Fig. 5 *B*, *right*, *Texas red positive*) were then counted. Similarly, the DNA-specific fluorophor bisbenzimide was added to the extract to visualize and count the total number of nuclei in the extract (Fig. 5 *B*, *left*). The result of these observations demonstrated that the nuclei fell into two distinct classes; those that had replicated (80%) and those that failed to replicate (20%). Importantly, the number of nuclei that replicated was identical to the number of nuclei added to the extract after dilution, and the number of nuclei that failed to replicate was identical to the number of nuclei assembled before dilution. Thus, by controlling the time of addition of chromatin relative to transport of cdk2–cyclin E, we can generate an extract which contains two sets of nuclei, one set representing pseudo G_2 nuclei which are inhibited for replication and one set representing G_1 nuclei which are able to carry out a single round of replication.

Like Cyclin E, Cyclin A also Inhibits DNA Replication and MCM3 Binding

In somatic cells, cyclin E is degraded during S phase of the cell cycle, and cyclin A becomes the predominant cyclin subunit associated with cdk2 (Dulic et al., 1992; Koff et al., 1992). Therefore, we have examined whether cyclin A, like cyclin E, can block replication in egg extracts. The extracts used in this study were all made in the presence of the protein synthesis inhibitor cycloheximide and lacked cyclin A. Therefore, recombinant *E. coli* produced GST–*Xenopus* cyclin A was added to the extract. This added protein rapidly associated with the endogenous pool of cdc2 to form active kinase (data not shown). Like cdk2–cyclin E, cdc2–cyclin A complex was efficiently transported into nuclei (data not shown; Pines and Hunter, 1991).

To determine if cyclin A inhibited DNA replication and MCM3 binding, cytosol was preincubated with 66 nM of cyclin A (30 min), and then sperm chromatin was added and the extract incubated an additional 30 min. After this, the sperm were separated from the cytosol by centrifugation through sucrose, and chromatin-bound MCM3 was determined both from Western blots probed with anti-MCM3 antibodies (Fig. 6 *B*) and immunofluorescent staining of the chromatin with this antibody (Fig. 6 *E*). The results of these experiments showed that in extracts preincubated with 66 nM cyclin A, both DNA replication in intact nuclei (Fig. 6 *A*) and MCM3 binding to chromatin (Fig. 6, *B* and *E*) were strongly inhibited. By contrast, when sperm were added to cytosol and incubated for 30 min before addition of 66 nM cyclin A, neither DNA replication (Fig. 6 *C*) nor MCM3 binding to chromatin (Fig. 6 *D*) was inhibited.

Cyclin A has been shown to be involved in both DNA replication and initiation of mitosis (Strausfeld et al., 1994, Jackson et al., 1995). Therefore, the inhibition of replication by cyclin A might occur because addition of cyclin A initiated mitosis. However, several pieces of evidence argue against this possibility. First, if cyclin A induced mitosis at 66 nM, it should do so independent of its order of addition relative to sperm. However, when cyclin A was added after sperm (*late addition*), DNA replication occurred normally (Fig. 6 *C*). Second, the morphological organization of both nuclei and DNA in extracts with 66 nM or higher cyclin A was examined by fluorescent microscopy. Nuclei formed, and the DNA within these nuclei decondensed in

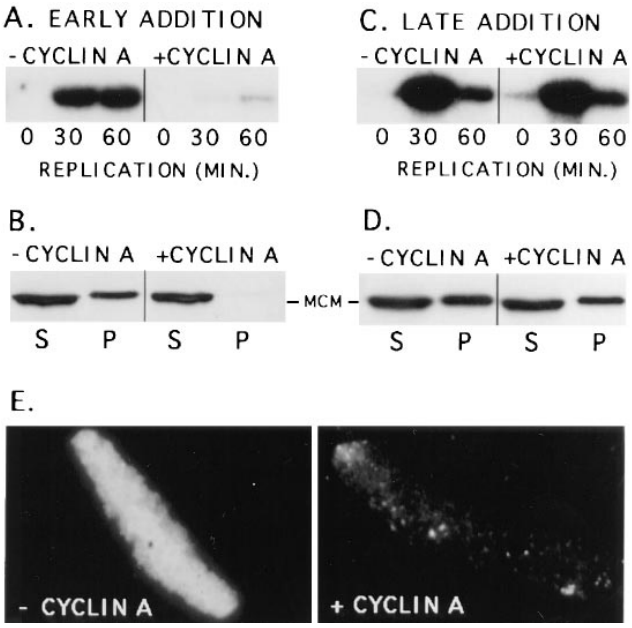

Figure 6. Cyclin A inhibits MCM3 binding to chromatin and blocks DNA replication. (*A*) Interphase cytosol was preincubated alone (− *CYCLIN A*) or with 66 nM of cyclin A–GST fusion protein for 30 min. After this preincubation, membrane and sperm (1,000 sperm/μl) were added. Equal volumes of these extracts were removed at the indicated times and labeled with [^{32}P]dATP for 15 min. In extracts lacking added cyclin A, replication occurred normally, while in the presence of cyclin A replication was strongly inhibited. (*B*) Interphase cytosol was preincubated with or without 66 nM of cyclin A-GST for 30 min. Sperm nuclei were then added to 5,000 sperm/μl. After a further 30-min incubation, the samples were diluted fivefold with ELB and the sperm chromatin separated from the cytosol by centrifugation through a sucrose cushion. The cytosol (*S*) and chromatin (*P*) fractions were analyzed for MCM3 content by Western blotting with anti-MCM3 antibody. Preincubation of cytosol with cyclin A strongly inhibited the subsequent association of MCM3 with chromatin. (*C*) Interphase cytosol was first incubated with 1,000 sperm/μl for 30 min. This reaction was then divided in half, and 66 nM cyclin A was added to one aliquot (+ *CYCLIN A*). Membrane was then added to both halves, and DNA replication was assayed as in (*A*). Under these conditions cyclin A did not inhibit DNA replication. (*D*) Interphase cytosol was incubated with 5,000 sperm/μl for 30 min. Either ELB buffer (− *CYCLIN A*) or 67 nM of cyclin A-GST (+ *CYCLIN A*) was then added to the reactions. After a further 30-min incubation, samples were collected and analyzed as in *B*. Under these conditions late addition of cyclin A did not prevent MCM3 from binding to sperm chromatin. (*E*) The samples were treated as in *B* above, however, instead of pelleting the sperm after incubation, the chromatin-bound MCM3 was visualized by staining with anti-MCM3 antibody, followed by rhodamine-labeled anti–rabbit IgG. Early addition of cyclin A (+ *CYCLIN A*) blocked the association of MCM3 with chromatin when compared to controls lacking cyclin A (− *CYCLIN A*).

extracts pretreated with cyclin A at concentrations up to 100 nM. In extracts pretreated with 200 nM cyclin A, mitosis was initiated based on the observation that the added sperm chromatin condensed into metaphase chromosomes and failed to form nuclei (data not shown). Thus, these results show that the inhibition of replication by addition of moderate concentrations of cyclin A does not occur because cyclin A causes the extract to enter mitosis.

Together, our experiments demonstrate that at the physiological concentration of cyclin A expected to be present in the nuclei of somatic cells during late S phase and G_2, cyclin A would not cause prebound MCM3 to be released from DNA. However, this concentration of cyclin A could strongly inhibit the reassociation of MCM3 with DNA once it had been released (Todorov et al., 1995) as a result of a single round of replication. As such, cdk2–cyclin A at this concentration, could block further rounds of DNA replication.

Discussion

The experimental results presented in this report strongly suggest that the transport of cdk2–cyclin E kinase complexes into nuclei may play a critical role in negatively regulating DNA replication during the cell cycle. In support of this we have shown that cdk2–cyclin E kinase is rapidly transported into nuclei formed in egg extracts. Further, by quantitating both the volume of nuclei and the amount of cdk–cyclin E transported per nucleus we show that this compartmentalization generates a cell cycle dependent gradient of cdk2–cyclin E within eggs. Before nuclear formation, the cdk2–cyclin E concentration in the cytosol is 200-fold lower than the concentration of cdk2–cyclin E present in nuclei. Our results strongly suggest that the low concentration of cdk2–cyclin E kinase present in the cytosol at the end of mitosis and before nuclei form allows MCM proteins to associate with chromatin, thereby potentiating DNA for replication. After nuclear formation, our data suggests that MCM proteins released during replication (Chong et al., 1995; Todorov et al., 1995) would be inhibited from rebinding chromatin due to the high cdk2 activity present in nuclei. In support of this, we have shown that increasing the concentration of cdk2–cyclin E in the cytosol 16-fold does not block assembly of sperm chromatin into nuclei but does inhibit both replication and the binding of MCM3. Importantly, we have also shown that this inhibition is dependent on the order of addition of cdk2–cyclin E relative to sperm chromatin. If cytosol is preincubated with cdk2–cyclin E before chromatin addition, MCM3 does not bind to chromatin, and DNA replication fails to occur. However, if MCM3 is allowed to bind to chromatin first, the subsequent addition of cdk2–cyclin E does not displace this bound MCM3, and DNA replication occurs normally. We have also shown that moderately high concentrations of cdc2–cyclin A kinase, like cdk2–cyclin E, prevents MCM3 from associating with chromatin and inhibits DNA replication but cannot displace prebound MCM3 from chromatin. This demonstrates that once MCM is bound to chromatin it cannot be displaced from the chromatin by increasing either cdk2–cyclin E or cdc2–cyclin A concentrations. Overall, these results suggest that during the embryonic cell cycle MCM proteins can only bind to and potentiate chromatin for DNA replication between the end of mitosis and before the assembly of chromatin into nuclei.

With respect to how cdk2–cyclin E blocks the binding of MCM3 to chromatin, a recent report in combination with the work presented here suggests that cdk2–cyclin E does not

inhibit the binding of MCM to chromatin by directly phosphorylating MCM. Specifically, Madine et al. (1995*a*) have shown that chromatin added to egg extracts depleted of MCM proteins form nuclei but do not replicate. However, when MCM proteins are added back to these extracts, the MCM complex is transported into preexisting nuclei and rescues DNA replication. If MCM proteins were substrates of cdk2 we would expect that the very high cdk2–cyclin E concentration within nuclei before addition of MCM would modify the added MCM as it entered nuclei, thereby blocking replication. Because this does not occur, direct phosphorylation of MCM by cdk2 does not appear to be critical for inhibiting its association with DNA. An alternative possibility would be that cdk2 kinase phosphorylates and inhibits protein factors which are required for the association of MCM with chromatin. It has recently been shown that both the multiprotein ORC complex and cdc6 protein must bind to chromatin before MCM proteins will associate with chromatin (Coleman et al., 1996). Our results demonstrate that cdk2–cyclin E inhibits the interaction of MCM3 with chromatin at a step downstream from the association of the ORC complex with chromatin. Specifically, we find that cdk2 concentrations which prevent MCM3 binding do not inhibit the association of ORC with chromatin. This suggests that proteins which both bind to chromatin after ORC and are required for the association of MCM with chromatin, such as cdc6, may be inactivated by cdk2 kinase activity. Interestingly, unlike the rescue of DNA replication by MCM, addition of cdc6 to nuclei formed in cdc6-depleted extracts does not rescue DNA replication (Coleman et al., 1996). It is quite possible that the high activity of cdk2 kinase in nuclei prevents cdc6 from binding to chromatin and this, in turn, prevents MCM from associating with chromatin.

A Role for cdk2 in Regulating DNA Replication during the Somatic Cell Cycle

Because cdk2–cyclin E is constituitively active during the embryonic cell cycle (Fang and Newport, 1991), our results suggest that MCM proteins can only bind to chromatin and potentiate this template for DNA replication before nuclear formation, when the local concentration of cdk2 kinase activity surrounding chromatin is relatively low. However, in somatic cells cdk2 kinase is inactive during early G_1 (Koff et al., 1991; Lew et al., 1991). Therefore, during early G_1 of the somatic cell cycle, MCM proteins should be able to enter nuclei and bind to chromatin. The activation of cdk2–cyclin E at mid-G_1 would be expected to block such binding during the remainder of the cell cycle. As such, the delayed activation of cdk2 kinase activity following mitosis may act to create a temporal window between the end of mitosis and mid-G_1 during which DNA is potentiated for replication. Consistent with this hypothesis, it has recently been shown that in *S. cerivisiae*, cdc6 synthesis can only potentiate DNA replication between anaphase and the activation of the metazoan equivalent of cdk2–cyclin E kinase, cdk1–Clb 5 and 6, at late G_1 (Piatti et al., 1996). The authors proposed that the activation of Cdk1-Clb kinases at late G_1 inhibited formation of replication competent DNA beyond this point. Our results strongly suggest that cdk2 activation at this time blocks replication by inhibiting the association of MCM with chromatin. This study also found that cdc6 associated with cdk1-Clb kinases, leaving open the question of whether cdk1-Clb complexes might inhibit cdc6 by sequestering the protein or by phosphorylating it. Our observation that the association of MCM3 with chromatin is sensitive to cdk2–cyclin E kinase activity and insensitive to the amount of cdk2–cyclin E complex present (Fig. 4) suggests that it is the kinase activity of cdk2 which blocks the association of MCM with chromatin.

A Relationship between cdk2-dependent Inhibition of Replication and Licensing Factor

It has been shown that when nuclei which have replicated once in a *Xenopus* extract are isolated, permeablized, and then added back to a second extract, these nuclei reinitiate replication (Blow and Laskey, 1988). By contrast, nuclei which are not permeablized before addition to a second extract fail to initiate replication. Similarly, when G_2 nuclei, isolated from somatic tissue culture cells, are permeablized and then added to egg extracts, they replicate, whereas intact G_2 nuclei do not (Leno et al., 1992). These observations have led to the proposal that a chromatin binding factor, called licensing factor, is essential for replication, and that the compartmental distribution of licensing factor between the nucleus and cytosol serves to limit replication to a single round per cell cycle (Blow and Laskey, 1988; Blow, 1993; Kubota and Takisawa, 1993). Specifically, the model predicts that licensing factor itself cannot enter nuclei. As such, the factor can only associate with and potentiate chromatin for DNA replication when the nuclear envelope is disassembled at mitosis. By contrast, our data strongly suggest that the compartmentalized accumulation of either cyclin E– or A–dependent cdk2 kinases within the nucleus may act to inhibit endoreduplication (Fig. 7). Our data are consistent with genetic observations implicating cdk kinases in blocking re-replication during the cell cycle (Broek et al., 1991; Hayle et al., 1994; Correa-Bordes and Nurse, 1995; Sauer et al., 1995). An advantage of this compartmentalized inhibitor model over the licensing model is that the binding of proteins required for one round of DNA replication is no longer restricted exclusively to mitosis. Rather, these associations can occur at times when cdk2–cyclin E– or A–dependent kinases are either dilute or inactive. During the early *Xenopus* embryonic cell cycle this would occur at the end of mitosis before nuclei have formed, while during the somatic cell cycle, this would occur during early G_1, when cdk2–cyclin E kinase is inactive.

For somatic cells in particular, the compartmentalized inhibitor mechanism is mechanistically more conservative and less restrictive than the licensing factor model. For example, in somatic tissues such as the liver, cells can remain in G_o of the cell cycle for one or more years before entering G_1 phase and then replicating their DNA. The "licensing" model would require that the license, which only associates with DNA at mitosis, remain completely stable during the one or more years that these cells are in G_o. Given the natural lifetime of proteins, it is likely that some of the license would be degraded during this period. As such, some of the DNA in these cells would be unable to

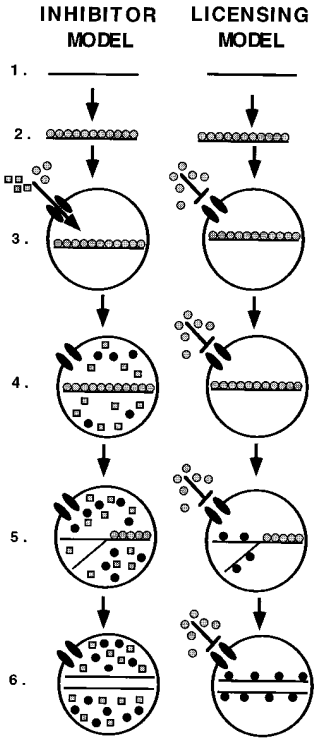

Figure 7. Comparison between the inhibitor and licensing models. In the inhibitor model, proteins required for potentiating DNA replication (*shaded circles*) bind to DNA when cdk2 activity is low. In *Xenopus* eggs this occurs at the end of mitosis before nuclear formation, when cdk2–cyclin E activity is dilute (*1* and *2*). In somatic cells this would occur during early G_1 when cdk2–cyclin E is inactive. After nuclear formation, cdk2–cyclin E (*black squares*) is rapidly transported into nuclei and accumulates (*4*). Replication potentiating proteins which enter the nucleus (*black circles*) are prevented from associating with DNA due to the high nuclear concentration of cdk2–cyclin E. However, the nuclear cdk2 does not displace potentiating proteins which are prebound to DNA. Similarly in somatic cells, activation of nuclear cdk2–cyclin E or A kinases during late G_1 would block any further potentiation of DNA for replication. During DNA replication the potentiating proteins are displaced from DNA and prevented from rebinding DNA by the presence of high concentrations of cdk2 kinase activity (*5* and *6*). In the licensing model, proteins (*shaded circles*) required for DNA replication associate with DNA at the end of mitosis (*1* and *2*). Because these proteins cannot enter the nucleus, enclosure of the DNA within the nucleus blocks further licensing (*3* and *4*). As a result of DNA replication the licensing proteins are converted to an inactive state (*5* and *6*).

replicate following release from G_o. By contrast, if proteins essential for potentiating replication, such as MCM3, are synthesized shortly after cells exit G_o, they could enter nuclei, bind to chromatin, and potentiate a round of replication during a well defined temporal window in which cdk2–cyclin kinases are inactive. Thus, the inhibitor model predicts that potentiation of chromatin for a single round of replication and inhibition of endoreduplication are controlled by the normal oscillations of cdk2–cyclin E and A activity, which occur during the cell cycle. We believe that the tight temporal linkage between the licensing event, the initiation of DNA replication, and the inhibition of re-replication supported by this model may provide a more conservative and controlled means of ensuring that all DNA is replicated once per cell cycle than the mitosis-dependent licensing mechanism.

Although our results strongly suggest that the accumulation of cdk2 kinase within nuclei can regulate the potentiation of chromatin for replication, it remains to be determined whether this is the only mechanism carrying out this function. Several observations indicate that this may not be the case. Specifically, we find that when Cip is added to extracts to inactivate cdk2 kinase activity before nuclear formation, MCM binding to chromatin is rescued (Fig. 4 *A*). However, after nuclear formation, inactivation of cdk2 by addition of Cip does not allow MCM to bind to chromatin (Hua, X., unpublished observations). This could occur because the accumulated cdk2 within nuclei rapidly activates a system that prevents MCM binding, and that once activated this system functions independently of cdk2. Alternatively, it may indicate that a second system exists to prevent MCM association, and that the activity of this second system is independent of the cdk2 concentration within nuclei. Resolving between these two possibilities will be an important focus of future investigation. However, given the essential nature of limiting replication to a single round per cell cycle, redundant cdk2-dependent and -independent mechanisms may be necessary to ensure cell viability.

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