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Regulated Chromosomal DNA Replication in the Absence of a Nucleus

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Summary

Using *Xenopus* egg extracts, we have developed a completely soluble system for eukaryotic chromosomal DNA replication. In the absence of a nuclear envelope, a single, complete round of ORC-dependent DNA replication is catalyzed by cytosolic and nuclear extracts added sequentially to demembranated sperm chromatin or prokaryotic plasmid DNA. The absence of rereplication is explained by an activity present in the nucleus that prevents the binding of MCM to chromatin. Our results indicate that the role of the nuclear envelope in DNA replication is to concentrate activators and inhibitors of replication inside the nucleus. In addition, they provide direct evidence that metazoans use the same strategy as yeast to activate DNA replication and to restrict it to a single round per cell cycle.

Introduction

In the last two decades, there has been much progress in elucidating the biochemical mechanism underlying eukaryotic chromosomal DNA replication. The discovery that SV40 viral DNA could be replicated in cytosolic extracts of HeLa cells (Li and Kelly, 1984) was used to identify many components of the eukaryotic replication fork (reviewed in Brush et al., 1995). Concurrently, *cis*-acting DNA sequences that serve as cellular origins of replication were discovered in the budding yeast *Saccharomyces cerevisiae* (reviewed by Newlon, 1988). These sequences were used to identify an origin recognition complex (ORC) whose binding to chromatin is a critical event in the initiation of DNA replication in all eukaryotes (reviewed by Stillman, 1996). Two other highly conserved factors, cdc6 and MCM, also bind to chromatin and participate in the initiation of DNA replication (Stillman, 1996).

In yeast, the outline of a model has emerged (frequently referred to as a "two-step" mechanism) that explains why origins of replication fire only once per cell cycle. In the first step, which occurs during early G1, a prereplicative complex (pre-RC) is established on chromatin by the sequential binding of ORC, cdc6, and MCM (Diffley and Cocker, 1992; Diffley et al., 1994; Aparicio et al., 1997; Donovan et al., 1997; Tanaka et al., 1997). In the second step, which defines the G1-S phase transition, cyclin-dependent kinases and cdc7/dbf4 kinase are activated, DNA replication is initiated, and cdc6 and MCM are lost from the origin (reviewed by Stillman, 1996). Importantly, S- and M-phase-specific cdks prevent the rebinding of MCM to chromatin, thereby preventing reinitiation until after the next mitosis (Dahman et al., 1995; Piatti et al., 1996; Tanaka et al., 1997; reviewed in Jallepali and Kelly, 1997). Aspects of this model have been observed in higher eukaryotes. Thus, in *Xenopus* egg extracts, the pre-RC is assembled immediately after mitosis and then disassembled during S phase (reviewed in Diffley, 1996). The mechanism by which rereplication is prevented during interphase of metazoans remains somewhat controversial (see below). However, there is some evidence that mitotic kinases prevent rereplication in higher eukaryotes (Usui et al., 1991; Sauer et al., 1995; Coverley et al., 1996), perhaps by blocking the binding of MCM to chromatin (Coue et al., 1996; Hendrickson et al., 1996; Mahbubani et al., 1997).

An intriguing question is to what extent the enzymology and regulation of DNA replication is dependent on higher-order nuclear structure. An important role for the nuclear envelope in activating DNA replication is suggested by experiments performed in *Xenopus* egg extracts (Lohka and Masui, 1983; Blow and Laskey, 1986; Newport, 1987), where any perturbation of the nuclear envelope disrupts DNA replication (Newport, 1987; Sheehan et al., 1988). A similar phenomenon was recently observed in a cell-free replication system that uses yeast nuclei incubated in yeast extracts (Pasero et al., 1997), suggesting that the requirement for a nuclear envelope in DNA replication is universal (see also Krude et al., 1997). Two functions of the nuclear envelope in potentiating DNA replication can be imagined (Newport, 1987). In one, the nuclear envelope generates, through selective nuclear transport, an intranuclear environment that is permissive for DNA replication (Leno and Laskey, 1991; Cox, 1992). In the second model, the nuclear envelope plays a more direct role in potentiating DNA replication. For example, it may be required to form an intranuclear skeleton or lamin network that in turn activates the replication machinery (Hozak et al., 1993; Moir et al., 1994; Spann et al., 1997).

The nuclear envelope has also been implicated in restricting DNA replication to a single round per cell cycle. It was found that nuclei that replicated once in interphase *Xenopus* egg extracts could undergo a second round of replication without an intervening mitosis if their membranes were transiently permeabilized (Blow and Laskey, 1988; Leno et al., 1992). This result has been interpreted in two ways. The licensing factor model proposes that the nuclear envelope prevents rereplication by excluding an activator of DNA replication from the nucleus (Blow and Laskey, 1988; Coverley et al., 1993; Laskey et al., 1996). In a model that is more similar to the yeast mechanism, it has been proposed that the nuclear envelope prevents rereplication by sequestering an inhibitor of rereplication, cdk2/cyclin E, within the nucleus (Hua et al., 1997). So far, it has not been possible to clearly distinguish between these two models.

We wanted to better understand what role the nuclear

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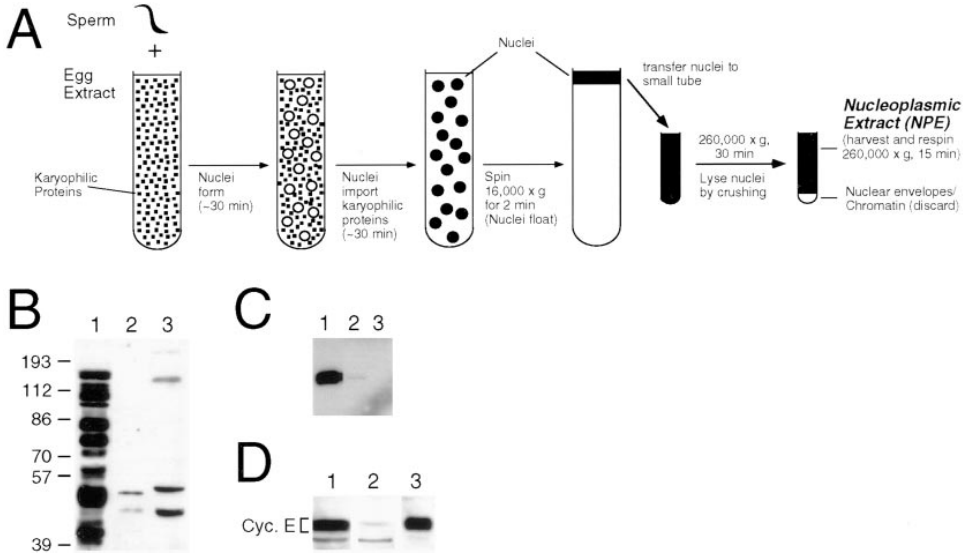

Figure 1. Preparation and Characterization of the Nucleoplasmic Extract

(A) Schematic diagram showing the procedure used to make NPE.

(B) Glycosylated membrane proteins are largely absent from NPE. 0.1 μl of purified membranes (lane 1), 1 μl of NPE (lane 2), and 1 μl of membrane-free cytosol (lane 3) were separated on 10% SDS–PAGE and analyzed by Western blotting using HRP-conjugated concanavalin A to detect glycosylated proteins.

(C) The amount of the membrane protein Ribophorin A in NPE is very low. The same Western blot as in (A) was probed with a monoclonal antibody against Ribophorin A (Meier et al., 1995).

(D) The cyclin E in an unfractionated egg extract (8 ml) is completely recovered in NPE (280 μl) prepared as in Figure 1. 1 μl of the nuclear assembly reaction before centrifugation (lane 1), 1 μl of the nonnuclear fraction after centrifugation (lane 2), and an equivalent fraction of NPE (0.035 μl) (lane 3) were analyzed by Western blotting using affinity-purified anti–cyclin E antibody.

envelope plays in activating DNA replication while preventing rereplication in *Xenopus* egg extracts. Our strategy was to mimic nuclear envelope formation in egg extracts by the addition of concentrated nucleoplasm and to determine whether chromosomal DNA replication still proceeded normally. This approach has allowed us to generate a completely soluble nuclear-free system for chromosomal DNA replication and to provide evidence that metazoans use the same "two-step" mechanism as yeast to regulate DNA replication during the cell cycle.

Results

Preparation of a Nucleoplasmic Extract from Synthetic Nuclei

Interphase *Xenopus* egg extracts assemble demembranated sperm chromatin into functional nuclei that carry out transport (Newmeyer et al., 1986) and undergo a single, complete round of DNA replication (Blow and Laskey, 1986; Newport, 1987). This extract can be fractionated to remove the membrane vesicles that form the nuclear envelope (Lohka and Masui, 1984; Newport, 1987; Sheehan et al., 1988). The resulting membrane-free egg cytosol supports the formation of pre-RCs containing ORC, cdc6, and MCM (Hua et al., 1997; Hua and Newport, 1998). However, these complexes are arrested at some step prior to the final events of initiation of replication. Adding back the purified membranes leads to the formation of an intact nucleus that undergoes DNA replication (Newport, 1987; Sheehan et al., 1988).

To determine whether the requirement for an intact nucleus could be circumvented, we prepared a highly concentrated nucleoplasmic extract (NPE). Briefly, demembranated *Xenopus* sperm chromatin was mixed with unfractionated *Xenopus* egg extract to form nuclei, the nuclei were harvested using a novel technique, and soluble nuclear components were extracted (Figure 1A). Using probes that recognize membrane proteins, we verified by immunoblotting that NPE contained very low concentrations of nuclear membranes (Figures 1B and 1C). To determine whether karyophilic proteins were recovered in NPE, we used immunoblotting to examine the distribution of cyclin E, a protein that was previously shown to be quantitatively transported into synthetic nuclei (Hua et al., 1997). Figure 1D shows that the cyclin E initially present in the egg extract was almost completely recovered in NPE (compare lanes 1 and 3). The final volume of NPE was about 4% of the volume of the egg extract used to form nuclei. Thus, using cyclin E as a measure, we estimate that soluble karyophilic proteins are about 25-fold more concentrated in NPE than in egg extract.

Sequential Addition of Egg Cytosol and Nucleoplasmic Extract to Sperm Chromatin Activates Genomic DNA Replication

We next tested whether NPE could support DNA replication. Demembranated sperm chromatin was mixed with NPE, [α-^{32}P]-dATP, and an ATP regenerating system and incubated for 90 min. Lane 2 of Figure 2A shows that there was no DNA replication in this reaction. Similarly,

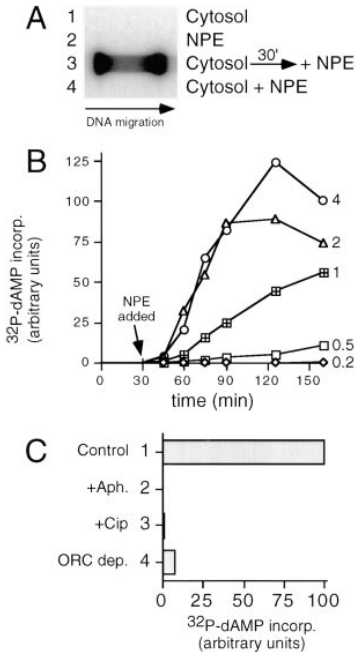

Figure 2. NPE Activates Genomic DNA Replication

(A) NPE only stimulates replication of sperm chromatin that has been preincubated in egg cytosol. Demembranated sperm chromatin was incubated with egg cytosol (lane 1), NPE (lane 2), egg cytosol for 30 min followed by the addition of 2.5 vol of NPE (lane 3), or with a 2.5:1 mixture of NPE:egg cytosol (lane 4) at a concentration of 1100/μl. After 90 min, the equivalent of 5000 sperm from each reaction was separated on an agarose gel (the direction of migration is from left to right) to measure the incorporation of [α-^{32}P]dAMP into high molecular weight DNA.

(B) NPE stimulates DNA replication in a dose-dependent fashion. Demembranated sperm chromatin was preincubated with cytosol in 10 μl aliquots. After 30 min, different amounts of NPE were added to each aliquot and the incorporation of [α-^{32}P]dAMP was measured using a phosphorimager and plotted in arbitrary units as a function of time. The numbers on the right indicate the amount of NPE added (in volumes relative to the egg cytosol). The concentration of sperm in each 10 μl starting aliquot of cytosol was adjusted so that after addition of NPE, the final concentration of sperm in each reaction was 400 /μl.

(C) NPE-stimulated DNA replication is eliminated by conditions that block genomic DNA replication. Sperm chromatin was preincubated for 30 min in egg cytosol at a concentration of ~1000/μl and then for a further 60 min in the presence of NPE (column 1). Column 2, both the cytosol and NPE contained 55 μg/ml aphidicolin. Column 3, the cytosol contained 1.1 μM His-tagged Cip1 (Guadagno and Newport, 1996) that became diluted to 440 nM after addition of NPE. In this experiment, Cip specifically blocked initiation because the same concentration of Cip had no effect on elongation in this system (data not shown). In column 4, the egg cytosol but not the NPE was immunodepleted with anti-Xorc2 serum (Walter and Newport, 1997). The relative amount of replication seen when immunodepletion was carried out with preimmune serum was 100.

no replication was detected when sperm chromatin was incubated with a mixture of NPE and membrane-free egg cytosol (lane 4), or egg cytosol alone (lane 1). However, when sperm chromatin was first incubated with egg cytosol for 30 min and then supplemented with NPE, a large amount of DNA replication was detected (Figure 2A, lane 3). Therefore, to activate replication, it is essential to expose the chromatin to cytosolic and nuclear environments in a sequential fashion.

Using this order-of-addition procedure, we tested how many volumes of NPE had to be added to 1 vol of cytosol in order to stimulate DNA replication. While the addition of 0.2 vol or less of NPE did not result in any detectable incorporation of [α-^{32}P]-dAMP (Figure 2B, diamonds), an abrupt increase in the rate and final amount of DNA synthesis was observed between 0.5 and 2 vol of added NPE (Figure 2B, compare open squares and triangles). Adding more than 2 vol of NPE did not lead to a significant further increase in replication (Figure 2B, circles and data not shown). Thus, NPE stimulates DNA replication in a saturable, dose-dependent fashion.

Measuring NPE-stimulated DNA replication under three different conditions confirmed that it corresponded to bona fide genomic DNA replication. First, addition of aphidicolin, which inhibits replicative DNA polymerases, completely abolished replication in NPE (Figure 2C, column 2). Second, 100-fold inhibition of replication was achieved by the addition of Cip protein (Figure 2C, column 3), which blocks the initiation of genomic DNA replication in *Xenopus* egg extracts through inhibition of cyclin E/cdk2 kinase (Strausfeld et al., 1994). Third, immunodepletion of ORC from egg cytosol led to a 12-fold reduction in the amount of DNA replication induced by the subsequent addition of NPE (Figure 2C, column 4). Significantly, although the cytosol was depleted of ORC, the added NPE was not and thus contained ORC (data not shown), but this ORC was not able to rescue replication. This result can be explained if NPE contains an inhibitor that prevents an early step in DNA replication (see below). In summary, the results in this section demonstrate that the ordered addition of cytosolic and nucleoplasmic extracts to sperm chromatin activates genomic DNA replication.

The Nucleoplasmic Extract Bypasses the Requirement for an Intact Nuclear Envelope in DNA Replication

Although NPE, like egg cytosol, contains very little membrane (Figures 1B and 1C), it was critical to prove directly that the replication of sperm chromatin in NPE was independent of a nuclear envelope. This was investigated using various criteria, the first of which was the morphology of the chromatin substrate. Sperm that were incubated in cytosol alone for 30 min appeared elongated when stained with Hoechst dye and lacked a nuclear envelope when viewed by phase-contrast microscopy (Figure 3A, I and II). When these sperm were incubated for a further 30 min following the addition of purified membranes, they acquired a clearly visible nuclear envelope and became large and round due to nuclear transport (Figure 3A, V and VI; Newport et al., 1990; Cox, 1992). In contrast, when the decondensed sperm were supplemented with NPE to initiate DNA replication, the shape of the sperm did not change significantly, nor did they acquire a phase-dense boundary (Figure 3A, III and IV), indicating that they lacked a nuclear envelope. Interestingly, addition of NPE did induce some changes in the fine structure of the sperm, as the chromatin became somewhat less homogeneously distributed (Figure 3A, compare I and III). The significance of this alteration is presently unclear.

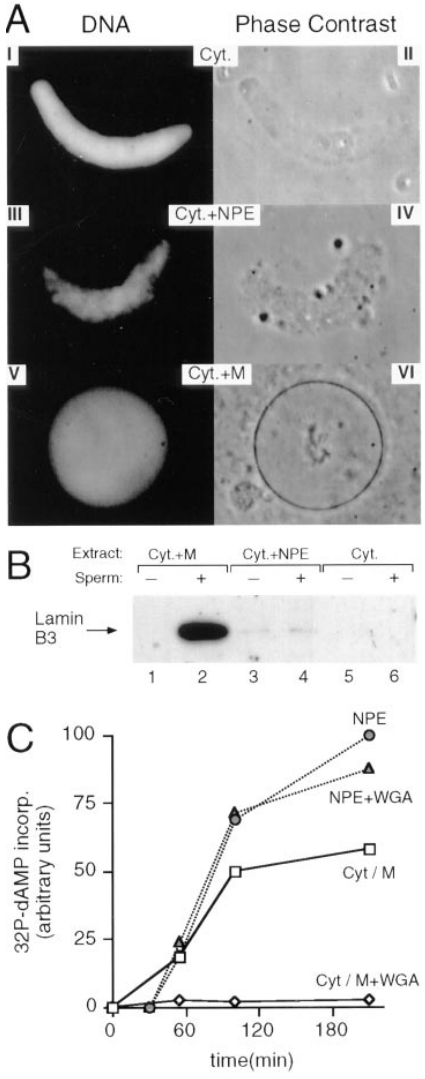

Figure 3. DNA Replication in NPE Occurs in the Absence of a Nuclear Envelope

(A) Sperm was analyzed by fluorescence microscopy to visualize DNA by a nonspecific dye, Hoechst (I, III, and IV), or by phase-contrast microscopy to visualize the nuclear envelope (II, IV, and VI) (Smythe and Newport, 1991). The sperm chromatin was incubated under three different conditions: 30 min in cytosol at 2000 sperm/μl (I and II); 30 min in cytosol, followed by an additional 30 min after adding 2 vol of NPE (III and IV); 30 min in cytosol, followed by an additional 30 min after adding 0.1 vol of purified membranes (V and VI).

(B) There are no lamins associated with sperm incubated in NPE. Sperm (16,000 total) incubated under the three conditions described in (A) was pelleted and washed gently. Proteins were stripped off the sperm with SDS sample buffer and analyzed by Western blotting using lamin B3 monoclonal antibody L_o46F7 (Benavente et al., 1985) (lanes 2, 4, and 6). Control reactions lacking sperm were analyzed in the same way (lanes 1, 3, and 5).

(C) NPE-stimulated DNA replication is independent of nuclear transport. 10 μl of cytosol at 1,800 sperm/μl was incubated for 30 min, then mixed with 3.5 vol of NPE, and replication was measured (circles). In another reaction, WGA was added to a final concentration of 0.5 mg/ml to cytosol and to NPE under otherwise identical conditions (triangles). To verify that the WGA was active, cytosol at 400 sperm/μl containing (diamonds) or lacking (squares) 0.5 mg/ml WGA was mixed with 0.1 vol of purified membranes, and replication was measured.

To further verify that the addition of NPE did not lead to the formation of a nuclear envelope, the sperm were isolated by centrifugation through a sucrose cushion and analyzed for the presence of lamin B3, a protein that is tightly associated with the nuclear envelope (Hutchison et al., 1994). Figure 3B shows that sperm incubated in NPE had no detectable lamin B3, and a dilution series of extract (not shown) showed that there was at least 300-fold less lamin protein bound to sperm incubated in NPE than in normal nuclei. This result demonstrates that no nuclear envelope has formed around the sperm incubated in NPE.

If replication in NPE occurs in the absence of a functional nuclear envelope, then this replication should be insensitive to inhibitors that block nuclear transport. To test this prediction, wheat germ agglutinin (WGA) was added to replication reactions containing either normal nuclei or sperm incubated in cytosol and NPE. WGA, which blocks transport into nuclei of proteins larger than 40–60 kDa (Finlay et al., 1987), completely inhibited DNA replication in normal nuclei (Figure 3C, compare squares and diamonds; Cox, 1992). In contrast, WGA had no effect on replication in NPE (Figure 3C, compare circles and triangles). Collectively, our results show that by incubating sperm chromatin sequentially in egg cytosol and nucleoplasmic extract, DNA replication can be activated in the absence of both a nuclear envelope and nuclear transport.

The Nucleoplasmic Extract Executes a Single, Complete Round of Semiconservative DNA Replication

Two important questions concerning the soluble replication system remained. First, how much of the input DNA is replicated? Second, is DNA replication in the soluble system limited to a single round, or is it unregulated? We addressed both questions by measuring the incorporation of bromodeoxyuridine (BrdU), a heavy analog of dTTP, into DNA under different conditions. First, we incubated sperm chromatin with egg cytosol and BrdU. After 30 min, the chromatin was isolated and separated on a CsCl gradient, and fractions of the gradient were analyzed by Southern Blotting using *Xenopus* genomic DNA as a probe. As expected, probe hybridization was detected in fractions corresponding to the density of DNA that has not incorporated any BrdU ("LL" DNA; Figure 4A). However, when the 30 min preincubation in cytosol was followed by the addition of NPE and a 3 hr incubation, 98% of the DNA migrated as hemisubstituted or "HL" DNA (Figure 4B; fractions 4–8). This demonstrates that essentially all the input DNA was replicated in a semiconservative manner. Identical results were observed with multiple preparations of NPE (data not shown).

The other important conclusion from this experiment was that there is no second round of DNA replication in the soluble system, despite the absence of a nuclear envelope. This was evident from the fact that no fully substituted ("HH") DNA was generated (Figure 4B). One possible explanation for the absence of HH DNA in this experiment was that the DNA became permanently inactivated during the first round of replication. To investigate whether this was the case, sperm chromatin

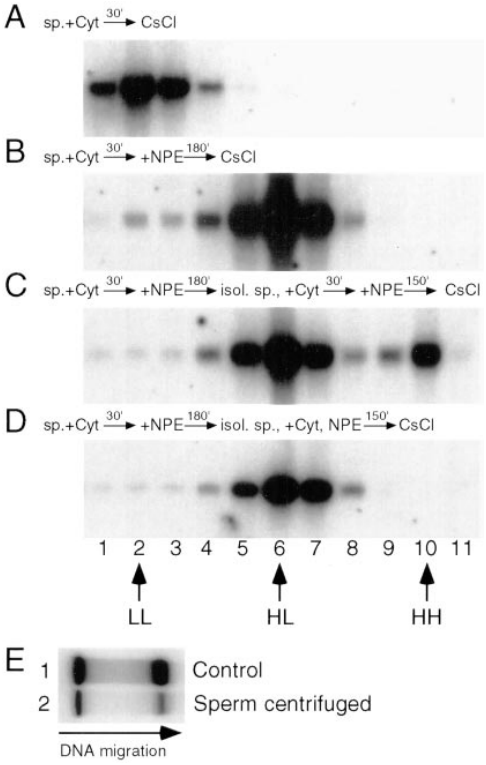

Figure 4. NPE Activates a Single, Complete Round of Semiconservative DNA Replication

(A–D) Sperm chromatin was incubated with egg cytosol at 4000/μl for 30 min, after which a sample was withdrawn for analysis (A). Subsequently, 2.5 vol of NPE was added and the reaction was incubated for a further 3 hr (B). The reaction was then split in two, layered over two sucrose cushions, and centrifuged to isolate the sperm. One chromatin pellet was mixed with fresh cytosol and incubated for 30 min, after which 2.5 vol of NPE was added and a 2.5 hr incubation was carried out (C). The other half of the sperm was incubated with fresh cytosol and NPE in a ratio of 1:2.5 and incubated for 2.5 hr (D). In all cases, cytosol and NPE contained 0.5 mM BrdU. Samples (A)–(D) were separated on CsCl gradients. Fractions were collected and analyzed by Southern blotting using *Xenopus* genomic DNA as a probe. LL DNA, 1.695 g/ml; HL DNA, 1.745 g/ml; HH DNA, 1.795 g/ml.

(E) Centrifugation inactivates 70% of the DNA template for replication. Sperm chromatin was incubated with egg cytosol for 30 min, followed by a 30 min incubation in NPE in the presence of 50 μg/ml aphidicolin to allow initiation and to prevent elongation. Subsequently, the chromatin was isolated by the same centrifugation procedure used in (C) and (D) above. Finally, cytosol and NPE (lacking aphidicolin) were added to the chromatin pellet in a ratio of 1:2.5, and the chromatin was allowed to replicate for 90 min (lane 2). As a control, sperm was incubated with egg cytosol for 30 min followed by a 90 min incubation after the addition of 2.5 vol of NPE (lane 1).

was isolated by centrifugation after the first round of replication had been completed. It was then incubated with fresh cytosol for 30 min, and NPE was added, followed by a further 2.5 hr incubation. As shown in Figure 4C, there was a DNA peak at the HH position in this experiment. Quantitation of the Southern blot showed that 12% of the DNA had been induced to undergo a second round of DNA replication. We suspected that the reason why a majority of the chromatin did not rereplicate was due to inactivation of the template during the centrifugation step. Indeed, when chromatin is centrifuged according to the protocol used in Figure 4C before the first round of replication, the amount of replication is 28% relative to a control that was not centrifuged (Figure 4E, compare lanes 1 and 2). This result explains why the majority of the chromatin in Figure 4C was not able to undergo a second round of replication.

We used this soluble system to investigate why replication is restricted to a single round in egg extracts. One explanation that would be consistent with the licensing factor model (Blow and Laskey, 1988) is that an essential initiation factor is used up during the first round of replication. If this was the case, then a second round of replication should be activated by transferring the chromatin to a fresh mixture of egg cytosol and NPE. To test this possibility, chromatin was allowed to go through one round of replication, and after isolating it by centrifugation, it was incubated in a mixture of fresh cytosol and NPE. The absence of any HH DNA (Figure 4D) shows that this treatment did not lead to a second round of replication. Therefore, it is not simply the consumption of an initiation factor during the first round of replication that prevents a second round of replication in the soluble system. Importantly, the only difference between the experiments shown in Figures 4C and 4D was that cytosol and NPE were added sequentially in 4C and simultaneously in 4D. Therefore, a second round of DNA replication can occur only if DNA is transiently exposed to a cytosolic environment, and then reexposed to the nuclear environment. Thus, the sequential incubation in cytosol and NPE is required for both the first (Figure 2A) and second rounds of DNA replication (Figure 4). This suggests that NPE contains an inhibitor that blocks the first and second rounds of DNA replication by similar mechanisms.

NPE Inhibits the Formation of Pre-RCs

We next addressed the mechanism by which NPE inhibits DNA replication. The fact that the inhibitory effects of NPE could be circumvented by preincubation in egg cytosol suggested that an NPE-sensitive step is carried out in the cytosol. It is known that egg cytosol supports the ordered binding of the initiation factors ORC, cdc6, and MCM to the chromatin to form pre-RCs (Coleman et al., 1996; Hua et al., 1997). Therefore, we decided to test whether NPE could interfere with the formation of pre-RCs. To do this, cytosol, sperm, and varying amounts of NPE were mixed together at the same time. After a 30 min incubation, the sperm chromatin was centrifuged through a sucrose cushion and analyzed by immunoblotting with antibodies against XMCM3, a subunit of the MCM complex (Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995). Strikingly, the addition of as little as 0.1 vol of NPE to 1 vol of cytosol led to a drastic reduction in the amount of MCM that loaded onto chromatin (Figure 5A, lane 3, upper panel). In contrast, rather than being inhibitory, the effect of NPE on ORC binding to chromatin was stimulatory (Figure 5A, lower panel). We next tested whether these chromatin templates were competent for DNA replication. Instead of isolating the chromatin by centrifugation as in Figure 5A, an amount of NPE was added that is expected to

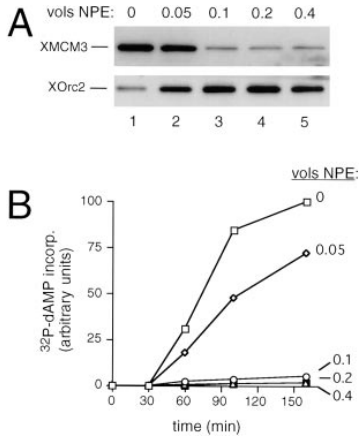

Figure 5. NPE Contains an Inhibitor of Replication that Blocks the Functional Association of MCM with Chromatin

Separate 10 μl aliquots of cytosol containing 2000 sperm/μl were mixed with different amounts of NPE (expressed as volumes; e.g., 0.4 vol is 4 μl) on ice and incubated for 5 min. Then, they were transferred to room temperature, and after a 30 min incubation, each reaction was supplemented with more NPE so that each reaction contained a total of 10 μl of NPE. Immediately after the second NPE addition, the chromatin from 10 μl of each reaction was isolated and analyzed by Western blotting to determine the amount of bound XMCM3 ([A], top panel) and XOrc2 ([A], bottom panel). The other 10 μl of each reaction was supplemented with [α-^{32}P]dATP and used to measure DNA replication (B).

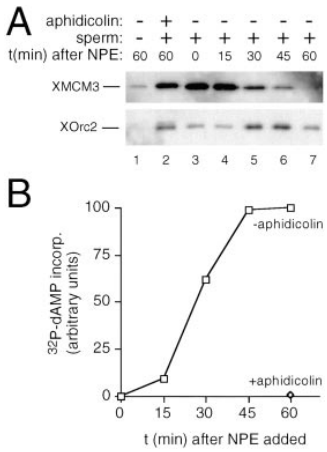

Figure 6. MCM Is Displaced from the Chromatin during DNA Replication in the Nucleus-Free System

(A) Sperm chromatin was incubated with cytosol at 3000/μl for 30 min and then mixed with 2 vol of NPE. Aliquots of 5000 sperm were isolated and analyzed by Western blotting using antibodies against XMCM3 (upper panel; Hua et al., 1997) and XOrc2 (lower panel; Carpenter et al., 1996; Walter and Newport, 1997). Chromatin samples were analyzed at 15 min intervals starting just before the addition of NPE (lanes 3–7). In controls, samples were analyzed after 60 min from reactions that contained 50 μg/ml aphidicolin (lane 2) or lacked sperm (lane 1).

(B) DNA replication was measured in the reactions described above containing (diamond) and lacking (squares) aphidicolin.

stimulate DNA replication from functional pre-RCs, and [α-^{32}P]dAMP incorporation was measured. The results show that the rate of DNA replication (Figure 5B) correlated well with the amount of MCM initially bound to the chromatin (Figure 5A). Therefore, an activity in NPE abolished the ability of MCM to functionally load onto chromatin, and this explains the negative effect of NPE on DNA replication. This result also explains why NPE, which contains ORC (data not shown), cannot rescue replication of chromatin incubated in ORC-depleted egg cytosol (Figure 2C).

MCM Is Displaced from Chromatin during DNA Replication in NPE

If an activity in NPE is to inhibit rereplication by blocking formation of prereplicative complexes, then these complexes must be disassembled during the first round of replication. It was shown previously that the MCM complex is displaced from chromatin during DNA replication in nuclei formed in egg extracts (Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995). To test whether this also occurs in the absence of nuclei, sperm chromatin was preincubated with egg cytosol, and NPE was added to initiate replication. To measure the amount of bound MCM complex, samples were removed at 15 min intervals and the chromatin isolated and analyzed for the presence of XMCM3. These experiments showed that most of the MCM that was associated with the chromatin at the time of NPE addition was released from the chromatin between 15 and 45 min after the addition of NPE (Figure 6A, lanes 3–6). Significantly, greater than 90% of the observed DNA replication also occurred during this time (Figure 6B, squares). When replication was inhibited with aphidicolin (Figure 6B, diamond), much less MCM was displaced from the chromatin (Figure 6A, compare lanes 2 and 7). In contrast to the behavior of MCM, ORC was not displaced from chromatin as a result of replication (Figure 6A, lower panel), in accordance with previous reports (Coleman et al., 1996; Romanowski et al., 1996; Rowles et al., 1996). Thus, as seen in nuclei, replication in NPE leads to the disassembly of pre-RCs. Since NPE also blocks the formation of pre-RCs (Figure 5), this result explains why no second round of DNA replication occurs in the soluble replication system.

Plasmid DNA Is Replicated Efficiently in NPE

It was important to measure the efficiency with which prokaryotic DNA sequences are replicated in the absence of nuclei, as it was previously found that they replicate, albeit very poorly, in conventional egg extracts (Blow and Laskey, 1986; Newport, 1987; Hyrien and Mechali, 1992; Mahbubani et al., 1992). Consistent with previous reports (Blow and Laskey, 1986), a 3 kb pBluescript plasmid replicated at a very slow rate in the conventional egg extract (Figure 7A, squares). In contrast, when plasmid DNA was preincubated in egg cytosol for 30 min and then supplemented with NPE, replication became detectable after 5 min and continued at a rapid rate for only 15–20 min before stopping. Like sperm chromatin, replication of plasmid DNA in the nucleus-free system was dependent on the presence of ORC (Figure 7B, compare lanes 1 and 2; 15-fold reduction) and on the sequential addition of cytosol and NPE to the DNA (data not shown). Figure 7C (bottom panel) shows that when replication of plasmid DNA in NPE was

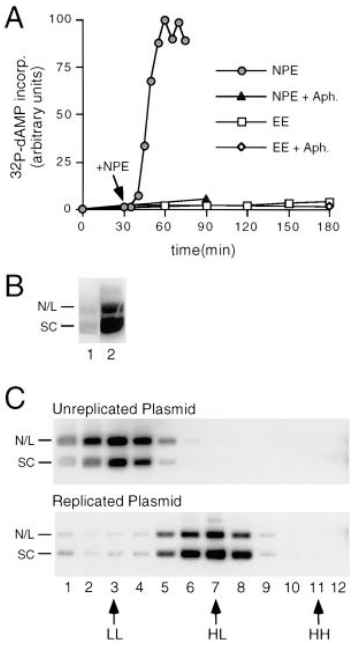

Figure 7. Plasmid DNA Replicates Efficiently in the Nucleus-Free System

(A) Kinetics of plasmid DNA replication in NPE and in unfractionated egg extract. Supercoiled pBluescript II KS(−) was incubated for 30 min at 2 ng/μl in egg cytosol containing (diamonds) or lacking (circles) 175 μg/ml aphidicolin. Following the addition of 2.5 vol of NPE, replication was measured after 90 min (triangle) or every 5 min for 45 min (circles). pBluescript II KS(−) was also incubated at 2 ng/μl in unfractionated egg extract (EE) in the presence (diamonds) or absence (squares) of 50 μg/ml aphidicolin. Replication was normalized to the concentration of input DNA and graphed in arbitrary units.

(B) Plasmid DNA replication in NPE is ORC-dependent. pBluescript II KS(−) was incubated in ORC-depleted egg cytosol (lane 1) or mock-depleted egg cytosol (lane 2) for 30 min at 4 ng/μl. The reaction was incubated for a further 60 min following the addition of 2 vol of undepleted NPE.

(C) CsCl equilibrium density centrifugation of plasmid DNA replicated in NPE. pBluescript II KS(−) was incubated for 30 min at 8 ng/μl (top panel) or 4 ng/μl (bottom panel) in 15 μl of egg cytosol. Subsequently, 2 vol of NPE containing (top panel) or lacking (bottom panel) 75 μg/ml aphidicolin was added, followed by a 60 min incubation in the presence of 0.5 mM BrdU. DNA was separated on CsCl gradients, and the DNA from each of 12 fractions from the gradient was analyzed by Southern Blotting using radioactively labeled pBluescript II KS(−) as a probe. LL, 1.708 g/ml; HL, 1.752 g/ml; HH, 1.80 g/ml. (N/L), nicked or linear DNA. (SC), supercoiled DNA

measured using density shift and Southern blotting, the vast majority (92%) of probe hybridization was found in the HL peak (fractions 5–8). The complete absence of a discrete peak in fraction 3 indicates that all of the DNA was replicated semiconservatively. Similarly, the absence of a discrete peak in fraction 11 indicates that none of the DNA underwent rereplication. These results confirm the hypothesis (Blow and Laskey, 1986) that the inefficient replication of plasmids in conventional egg extracts is due to inefficient assembly of nuclei around these substrates. In addition, they show that chromosomal DNA replication can be reconstituted from entirely soluble components.

Discussion

Current cell-free systems that support eukaryotic chromosomal DNA replication require the presence of intact nuclei (Newport, 1987; Pasero et al., 1997; see also Krude et al., 1997). Using *Xenopus* egg extracts, we have developed a completely soluble nucleus-free system for chromosomal DNA replication in eukaryotes. Pre-RCs are formed on sperm chromatin or plasmid DNA in a cytosolic *Xenopus* egg extract. The subsequent addition of a concentrated nucleoplasmic extract (NPE) leads to the initiation of a complete round of DNA replication and the dissociation of MCM from chromatin. The absence of rereplication is explained by the fact that NPE prevents the rebinding of MCM to chromatin. Therefore, the nucleus-free system that we have developed is analogous to the two-step mechanism shown to operate in yeast (see Introduction). These similarities make the nucleus-free system a powerful biochemical model to understand the steps that lead to the regulated duplication of the genome.

The Role of Higher-Order Nuclear Structures in Activating DNA Replication

We have shown that, with regard to supporting DNA replication, the formation of a nucleus can be functionally replaced by a concentrated nuclear extract. We conclude that the role of the nuclear envelope in previous cell-free systems (Newport, 1987; Pasero et al., 1997) and in living cells is to generate a soluble intranuclear environment that is permissive for DNA replication. Does this role involve concentrating activators of replication inside the nucleus or excluding cytosolic inhibitors of replication from the nucleus? Because replication in the nucleus-free system proceeds in the presence of significant amounts of cytosol (Figure 2B), we can rule out the latter model. Thus, it appears that the envelope concentrates activators of DNA replication within nuclei to a level where they become functional. This model is supported by our observation that DNA replication is only rapid and complete above a specific threshold concentration of NPE (Figure 2B).

Besides the nuclear envelope, are there other aspects of nuclear architecture that are required to stimulate DNA replication? In mammalian nuclei, lamins colocalize with sites of DNA replication (Moir et al., 1994), and disruption of lamin structure in egg extracts inhibits DNA replication in nuclei (Newport et al., 1990; Jenkins et al., 1993; Spann et al., 1997). These observations suggested that lamins may be required to catalyze DNA replication, perhaps as a structural support for DNA replication centers. Arguing against this model, we could not find any lamin B3 associated with sperm chromatin undergoing replication in the nucleus-free system (Figure 3B). In addition, we have immunodepleted ~90% of lamin B3 from egg cytosol and NPE without any effect on DNA replication in the nucleus-free system (J. W. and J. N., unpublished data). We suggest that the apparent requirement for lamins in replication may have been an indirect effect resulting from the destabilization of the nuclear envelope that is observed whenever the function of the lamins is disrupted (Newport et al., 1990; Jenkins

et al., 1993; Spann et al., 1997). In summary, we have found no evidence for the involvement of higher-order structures in catalyzing DNA replication. Further work will be required to determine whether any such structures participate in DNA replication.

The Role of the Nuclear Envelope in Restricting DNA Replication to a Single Round

In yeast, S phase–specific cdks prevent rereplication by blocking the rebinding of MCM to chromatin after it is displaced during the first round of replication (Tanaka et al., 1997; reviewed in Jallepali and Kelly, 1997). Similarly, in egg extracts, MCM dissociates from chromatin during DNA replication and then is unable to rebind for the remainder of S phase (Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995; Hua and Newport, 1998). What prevents MCM from rebinding to chromatin in egg extracts? In this paper, we have demonstrated that the nucleus contains an activity that actively blocks the binding of MCM to chromatin while having no effect on the binding of ORC. Significantly, a previous report from our laboratory showed that when recombinant cyclin E/cdk2 is added to egg cytosol at high concentrations in the absence of nuclei, the binding of MCM is blocked while ORC is unaffected (Hua et al., 1997). Based on this previous observation, and the fact that the concentration of cyclin E/cdk2 in NPE is very high (Figure 1 and data not shown), it is likely that the inhibitor of MCM binding in NPE is cyclin E/cdk2. Thus, our results directly support the model (Hua et al., 1997) that the nuclear envelope restricts DNA replication to a single round through an inhibitor of MCM binding that is concentrated within the nucleus during interphase. According to this model, the inhibitor is diluted after the nuclear envelope breaks down in mitosis, allowing the renewed binding of MCM to chromatin.

It is important to discuss the implications of our results for the licensing factor model (Laskey et al., 1996). This model was based on the observation that in egg extracts, G2 nuclei that have replicated once will replicate again if their membrane is permeabilized and then repaired (Blow and Laskey, 1988; Leno et al., 1992). The fact that G2 nuclei do not rereplicate if they are permeabilized and repaired in buffer before transfer to fresh extract was taken as evidence that permeabilization normally allows entry of an activator rather than exit of an inhibitor (Coverley et al., 1993). However, these results can be equally well explained by a model in which the nucleus is required to concentrate an inhibitor that prevents binding of MCM to pre-RCs. In this view, G2 nuclei that have been permeabilized and repaired in buffer lack pre-RCs (because they have replicated) and they are devoid of the inhibitor (because they were permeabilized). As such, when these nuclei are added to fresh extract, we expect that there is a kinetic competition between the import of the inhibitor and the assembly of functional pre-RCs containing MCM. We suggest that the inhibitor reaches a critical intranuclear concentration before MCM can bind, and that this explains the absence of rereplication. Consistent with this model, we find that a very low concentration of NPE is sufficient to prevent MCM binding to pre-RCs (Figure 5B). Moreover, we have demonstrated directly that there is no second round of replication in the nucleus-free system even when fresh replication factors are added after the first round of replication has been completed (Figure 4D). This observation argues forcefully that exclusion of a replication protein from the nucleus is inadequate to explain the block to rereplication in egg extracts. In summary, the inhibitor model appears sufficient to explain all the data relating to rereplication in egg extracts.

Specific Origin Sequences Are Not Required for Efficient, Regulated DNA Replication

Previous studies have shown that prokaryotic plasmids injected into *Xenopus* eggs (Harland and Laskey, 1980; Mechali and Kearsey, 1984) or incubated in *Xenopus* egg extracts (Blow and Laskey, 1986; Newport, 1987; Hyrien and Mechali, 1992; Mahbubani et al., 1992) replicate semiconservatively by initiating at random DNA sequences (see also Hyrien and Mechali, 1993). These studies raised the possibility that DNA replication in metazoans might initiate at random sites. However, the interpretation of these experiments was limited by the fact that when compared to genomic replication, plasmid replication was very inefficient. We have now shown that when the requirement for intact nuclei is circumvented with NPE, prokaryotic plasmid DNA replication is very efficient, properly regulated, and ORC-dependent. Mapping the location of nascent DNA strands by restriction digestion confirmed that initiation occurs at multiple sites on the plasmid (L. S. and J. N., unpublished data). These findings have several important implications. First, unlike sperm chromatin, the size and sequence of plasmids can be extensively manipulated, adding an important new variable to in vitro studies of replication. In addition, by showing that prokaryotic plasmids support highly efficient and properly regulated DNA replication, we strongly support the earlier studies that suggested that chromosomal DNA replication in eggs initiates at random sequences. Moreover, our finding that plasmid DNA replication requires ORC indicates that sequence-independent DNA replication is mechanistically similar to DNA replication in somatic cells. We further note that DNA replication during early embryogenesis must occur with very high speed and fidelity if the organism is to develop normally. Therefore, we now see no a priori reason why DNA replication could not also initiate from random sequences in somatic cells where the requirements for speed and fidelity should be more relaxed. This question clearly requires further study given the current lack of conclusive evidence for or against sequence-specific initiation in somatic cells of metazoans.

Experimental Procedures

Extract Preparation

Unfractionated egg extracts, purified membranes, and membrane-free egg cytosol were prepared essentially as described previously (Smythe and Newport, 1991). To make NPE, unfractionated egg extract was supplemented with nocodazole to a concentration of 3.3 μg/ml and recentrifuged at 16,000 × g and 4°C in an HB-4 rotor for 10 min. The viscous dark brown layer at the top of the tube was completely removed by aspiration. 4.5 ml of extract was decanted into a fresh 2063 Falcon tube and supplemented with an ATP regeneration system and demembranated sperm chromatin (Smythe and

Newport, 1991) at a concentration of 4000/μl. The mixture was incubated at room temperature for 70–80 min. The reaction was then centrifuged at 16,000 × g and 4°C in an HB-4 rotor for 2 min. The clear ~2 mm thick layer of nuclei was gently removed from the top of the tube and transferred to Beckman 5 × 21 mm ultracentrifuge tubes. The nuclei were supplemented with ELB (lacking DTT and cycloheximide) to a final volume of 225 μl (assuming a 4.5 ml nuclear assembly reaction), mixed thoroughly, and centrifuged in a Beckman TL100 ultracentrifuge using a TL55 swinging bucket rotor furnished with teflon adapters (Beckman). Centrifugation was for 30 min at 55,000 rpm (260,000 × g) and 2°C. After centrifugation, any lipids at the top of the sample were carefully aspirated with a pulled Pasteur pipette, and the nucleoplasmic extract was harvested. The extract was respun for 15 min in the TL55 rotor as above. The final volume of NPE was ~180, and it was frozen in liquid nitrogen as one-use aliquots. A detailed protocol is available upon request.

DNA Replication Reactions

In Egg Cytosol + NPE

Cytosol was supplemented with an ATP regenerating system, nocodazole (3.3 μg/ml final concentration using a 0.5 mg/ml stock made in DMSO), and ~0.1 μCi/μl [α-^{32}P]dATP and incubated with the desired concentration of sperm chromatin for 30 min. Subsequently, NPE (supplemented with the ATP regenerating system) was added, and the reaction was immediately divided into 3 μl aliquots. The reactions were stopped and DNA replication measured using agarose gel electrophoresis (Dasso and Newport, 1990) and a Molecular Dynamics phosphorimager.

In Cytosol + Membranes

The cytosol and sperm mixture used for replication with NPE was supplemented with 0.1 vol of purified membranes, and replication was analyzed as above.

In Unfractionated Egg Extract

This extract was supplemented with the ATP regenerating system, 3 μg/ml nocodazole, ~0.1 μCi/μl [α-^{32}P]dATP, and DNA.

BrdU Substitution and Southern Blotting

After the incubation in egg cytosol or cytosol + NPE (see Figures 4A and 4B, respectively), the reaction was mixed with 1 vol of ELB and centrifuged for 1 min through the sucrose cushion described under "chromatin binding assay," and the supernatant was removed. To isolate the sperm chromatin after the first round of DNA replication (see Figures 4C and 4D), the reaction was mixed with 1 vol of ELB and then centrifuged through a sucrose cushion as in Figures 4A and 4B. After removal of the supernatant, cytosol and NPE were added sequentially or together into the same tube (because sperm cannot be removed from the tube) and incubated. Subsequently, 200 μl of ELB was added and the sample centrifuged for 1 min; the supernatant was removed, 200 μl of ELB (containing 0.5 M sucrose) added, the sample centrifuged again for 0.5 min, and the supernatant removed. Samples A–D were mixed with 100 μl of Buffer A (5 mM EDTA, 20 mM Hepes [pH 7.6], 50 mM NaCl), containing 0.5 mg/ml Proteinase K, and 0.5% SDS. Following a 60 min incubation at 37°C, samples were phenol/chloroform-extracted, mixed with 12.8 ml of 1.75 g/ml CsCl dissolved in TE, and centrifuged at 30,000 rpm in a Beckmann Ti70.1 rotor for 44 hr. Twelve 1 ml samples were collected, and 150 μl of each sample was diluted 2-fold with water, and ethanol-precipitated in the presence of 20 μg of carrier RNA. Samples were then separated on a 0.8% agarose gel and analyzed with a sensitive Southern blotting procedure (Walter et al., 1994) using a probe that was prepared by random priming of *Xenopus* genomic DNA.

To analyze plasmid DNA replication using BrdU substitution and Southern blotting (Figure 7A), replication reactions were mixed with 1 vol of buffer A containing SDS and Proteinase K, digested, phenol/chloroform- and chloroform-extracted, ethanol-precipitated in the presence of 20 μg of carrier RNA, and resuspended in 100 μl of TE. The CsCl gradient (see above) was centrifuged for 70 hr.

Chromatin Binding Assay

To purify sperm chromatin, up to 10 μl of extract containing sperm was withdrawn from a reaction at the desired time, brought to 50 μl with cold ELB, and layered over a 170 μl ELB cushion (containing 0.5 M instead of 0.25 M sucrose) in 5 × 44 mm microcentrifuge tubes (Beckman). The sample was centrifuged in a Beckman E microcentrifuge using a horizontal rotor at 16,000 × g for 20 s at 4°C. Most of the supernatant was aspirated, and the pellet was washed gently with 200 μl ELB and respun in the same tube for 30 s as described above. All but ~3 μl of the supernatant was carefully removed, and the sample was boiled in 10 μl of SDS sample buffer.

Miscellaneous

The Ribophorin A antibody was a generous gift of Dr. David Meyer. Concanavalin A–horseradish peroxidase (ConA-HRP) was obtained from E. Y. Laboratories, Inc., San Mateo, CA, and it was used in a 1:20 dilution. The anti–cyclin E antibody was raised in rabbits against a full-length cyclin E His-tag fusion protein. It was affinity-purified on full-length cyclin E protein immobilized on nitrocellulose membrane. ~99% of ORC was removed by three consecutive immunodepletions using anti-Xorc2 antiserum as described previously (Walter and Newport, 1997). Signals on Western blots were detected by enhanced chemiluminescence using horseradish peroxidase secondary antibodies. pBluescript II KS(−) was purified using the alkaline lysis method and equilibrium density centrifugation on CsCl gradients. The supercoiled band was isolated and repurified using a Qiagen QIAquick gel extraction kit.

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