An Analysis of the Regulation of DNA Synthesis by cdk2, Cip1, and Licensing Factor

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Abstract. The activation of DNA replication appears to involve at least four steps. These include origin recognition, origin unwinding, primer synthesis, and a switching step to a continuous elongation mode. Moreover, in higher eukaryotes a number of studies have shown that much of the DNA replication which occurs is restricted to specific sites within the nuclei. It has been proposed that these replication foci are composed of a large number of origin sites which are clustered together into an aggregate. The molecular basis for this aggregation is currently not well understood. Regulation of the activation of DNA replication is a complicated process. The G1-S kinase cdk2 is a positive regulator of replication. The p21 protein is a negative regulator of replication both by inhibiting cdk2 kinase and the replication protein PCNA. Moreover, it has been proposed that origin usage is restricted to a single firing per cell cycle by a "licensing factor." Using a cell-free replication system derived from Xenopus eggs we have investigated at what step in the replication process these regulators participate.

We present evidence that the clustered organization of DNA into foci is not a transient arrangement, but rather, it persists following DNA replication. We also find that foci form on both sperm chromatin and bacteriophage lambda DNA incubated in extracts depleted of cdk2 kinase. Therefore, our data support the conclusion that organization of chromatin into foci is an early event in the replication pathway preceding activation of cdk2 kinase. With respect to the role of cdk2 during activation of DNA replication we find that in cdk2-depleted extracts primer synthesis does not occur and RP-A remains tightly associated with foci. This strongly suggests that cdk2 kinase is required for activating the origin unwinding step of the replication process. Consistent with this interpretation we find that addition of rate limiting quantities of the cdk2 inhibitor p21 protein to an extract delays primer synthesis. Interestingly, in the presence of p21 primer synthesis does occur after a delay and then replication arrests. This is consistent with the published demonstration that p21 can inhibit PCNA, a protein required for replication beyond the priming step. Therefore, our results provide additional support to the proposal that the post-priming switching step is a key regulatory step in replication. With respect to the role of licensing factor during DNA replication it has recently been shown that treatment of mitotic extracts with kinase inhibitor DMAP inactivates "licensing factor." As such, when these treated extracts enter S-phase they are unable to replicate DNA. By following biotin-dUTP incorporation and RP-A distribution in such DMAP-treated extracts we have determined at what step replication arrests in the absence of licensing factor. Surprisingly, we find that licensing deficient extracts, like p21-treated extracts, arrest following primer synthesis. This indicates that licensing factor may not be necessary for origin opening and primer synthesis, but rather, is essential in the switching step which converts replication from a priming to the elongation mode.

ACCURATE replication of the genomic DNA during S-phase of the cell cycle is an essential event required to maintain cell viability. Much of what we currently know about the DNA replication process in eukaryotic cells derives from a reconstituted in vitro system using the SV-40 viral origin of replication, viral large T antigen, and proteins purified from cell extracts. Based on studies using this model system, it has been demonstrated that DNA replication can be divided into several steps (Waga and Stillman, 1994). In the first step an initiator protein (large T antigen) recognizes and binds to the viral origin sequence to form a latent pre-initiation complex. Subsequently the origin sequence is stably opened by the combined action of large T antigen and the single-stranded DNA binding protein RP-A. The unwound

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complex generated by this step is then used as template by polymerase α to synthesize a 100–200 nucleotide long primer first on the leading-strand and then on the lagging strand. Following the synthesis of these primers further elongation appears to be dependent on a switching event in which DNA polymerase δ replaces DNA polymerase α as the enzymatic activity responsible for further synthesis. This switching event requires the activities of two replication proteins, RPC and PCNA, which stimulate the processivity of polymerase δ. Thus, replication of the SV-40 genome consists of at least four steps: recognition of specific origin sequences, origin unwinding, primer synthesis, and switching.

It is now clear that in many aspects SV-40 DNA replication accurately reflects the process responsible for genomic replication in eukaryotes. For example, replication of the SV-40 template occurs as a result of specific interaction between large T antigen and SV-40 origin sequence. Similarly, in yeast well-defined origin sequences have also been identified and an origin recognition complex (ORC)[1] consisting of six proteins binds these sequences (Bell and Stillman, 1992). Despite these similarities, it is likely that the increased complexity of higher eukaryotic genomes, which are approximately 10^6 times larger than the SV-40 template, has led to modifications not represented by the SV-40 system. For example, in higher eukaryotes sequence-specific origins have proven to be frustratingly elusive to identify despite numerous serious attempts (for review see DePamphilis, 1993). In fact, small prokaryotic plasmids are initiated at random sites even though still replicated under cell cycle control when injected into Xenopus eggs (Harland and Laskey, 1980; Mechali and Kearsey, 1984; Hyrien and Mechali, 1992; Mahbubani et al., 1992). Moreover, if specific origin sequences do function during replication of higher eukaryotic DNA, then origin usage may be developmentally regulated as suggested by the much smaller replicon sizes in embryonic than somatic cells of many organisms (Blumenthal et al., 1973). One explanation for this apparent inconsistency might be that in higher eukaryotes many potential origin sequences exist but only a subset is actually selected for usage in each cell cycle. As to the mechanism by which sequences might be selected, a large number of studies have demonstrated that much of the DNA replication in higher eukaryotic cells occurs at discrete foci within the nuclei (Nakamura et al., 1986; Nakayasu and Berezney, 1989; Mills et al., 1989; Cox and Laskey, 1991). It is estimated that these foci are made up of 300–1,000 replicon units (Mills et al., 1989). Thus, initiation of replication in higher eukaryotes may be restricted to origins that are directly associated with such replication foci. The protein composition and organization of DNA around these foci are not well understood. However, in a recent study using Xenopus egg extracts and demembranated sperm chromatin, Adachi and Laemmli (1992) have shown that the replication protein RP-A can associate with 100–300 discrete foci before nuclear envelope formation. Since replication first occurs at these foci, they may correspond to the SV-40 pre-initiation complex but in an aggregated state (estimated at 300-1,000 origins per center).

1. *Abbreviations used in this paper*: GST, glutathione S transferase; ORC, origin recognition complex; 6-DMAP, 6-dimethylamino-purine.

During S-phase of the cell cycle in SV-40-infected cells, the SV-40 DNA template is replicated multiple times. By contrast, genomic DNA is replicated only once per cell cycle. Therefore, SV-40 replication lacks the regulatory mechanism that prevents endo-reduplication of genomic DNA. Regarding how this regulatory system works, it has been proposed that a "licensing factor" which lacks a nuclear localization signal binds to chromatin at mitosis prior to nuclear envelope formation (Blow and Laskey, 1988). This licensing factor prevents endo-reduplication by being both necessary for replication and at the same time inactivated by replication. Thus once the license on a segment of DNA has been used to replicate DNA, further replication of this segment is blocked. Recently two reports have implicated a kinase activity as playing an important role in activating licensing factor at the end of mitosis. Specifically it has been shown that when mitotic Xenopus egg extracts are treated with kinase inhibitors and then induced to enter S-phase, these extracts are unable to carry out DNA replication in a manner consistent with the absence of a licensing factor (Blow, 1993; Kubota and Takisawa, 1993). Although these reports clearly demonstrated that the kinase inhibitor-treated extracts were blocked for DNA replication, it was not determined whether this block occurred before initiation or at a step following initiation and primer synthesis.

In actively proliferating cells, the activation of cdk2 (or cdc2/cdc28 in yeast) kinase at the G1-S phase transition has been shown to play a critical role in activating DNA replication (for review see Roberts, 1993). Depletion of cdk2 from a Xenopus in vitro replication system prevents replication (Fang and Newport, 1991) and inhibition of cdk2 kinase activation in living cells prevents cells from entering S-phase (Pagano et al., 1993). In principle, cdk2 could control replication by regulating any one of the four steps outlined above which are essential to establishing a functional replication complex. For example, investigations using the in vitro SV-40 replication system have shown that origin unwinding is much more active when cellular replication proteins are extracted from S-phase cells than from G1 cells (Roberts and D'Urso, 1988). Recently, a second regulatory step involving the Cip1 protein has been identified using the SV-40 system. Cip1 (also called p21, Sdi1, Waf1, CAF20, and Pic1) was initially isolated as an inhibitor of cdk2 kinase activity (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993). However, in a SV-40 replication system reconstituted with purified components but lacking cdk2, Cip1 can bind directly to PCNA and inhibit the PCNA-dependent switching step required for converting replication from the priming to elongating mode (Waga et al., 1994). Together these examples demonstrate that distinct steps in the replication pathway can be regulated. Moreover, they suggest that regulation of DNA replication during S-phase may involve multiple regulatory elements that have yet to be characterized.

In this report we have used RP-A and biotin-labeled dUTP as two markers to study the regulation of DNA replication in Xenopus egg extracts. Our results show that cdk2 is not required for the assembly of RP-A foci but is essential for initiation of replication. Moreover, using the kinase inhibitor 6-dimethylamino-purine (6-DMAP) we have been able to arrest DNA replication at the switching step following primer synthesis. Thus, our results indicate that the switching step between priming and elongation may be another highly regu-

lated process essential for maintaining control over replication during S-phase. We also show that the Cipl protein can inhibit DNA replication at both the cdk2-dependent step and the 6-DMAP-sensitive step.

Materials and Methods

Extract Preparation and Manipulation

Crude interphase extracts and further fractionation of these extracts into cytosol and membrane were performed as described by Smythe and Newport (1991). CSF extracts were made according to Murray (1991) except with the modification that eggs were crushed at 4°C and extracts were routinely activated with 0.4 mM $CaCl_2$. P13 depletion was done according to Pfaller et al. (1991). Briefly, p13 beads were incubated in extracts with agitation at 4°C for 1 h, and then removed by brief centrifugation. CNBr-activated Sepharose beads blocked by ethanolamine were used as the mock control. For RP-A depletion, protein A-Sepharose beads (Sigma Chem. Co., St. Louis, MO) were first coated with the rabbit anti-RP-A polyclonal antibody (Fang and Newport, 1993). The extract was then depleted in a procedure similar to that used for p13 depletion. Protein A-Sepharose beads alone were used as a control.

Replication Reactions

Sperm nuclei were usually assembled at 500 sperm/μl extract as described by Smythe and Newport (1991). Nuclei formed around lambda DNA were assembled by mixing 0.1 μg lambda DNA (pre-heated at 65°C for 5 min and then cooled slowly at 23°C for 10 min; Newport, 1987) with 10 μl crude interphase extract. Reactions were incubated at room temperature for various times. For visual replication assays 10 μM biotinylated dUTP (Boehringer-Mannheim Corp., Indianapolis, IN) was included in the reaction. For M13 replication assays, 5 ng M13 single-stranded DNA and 0.01 mCi ^{32}P-dCTP (ICN Radiochemicals, Costa Mesa, CA) were added to 20 μl extracts. Following incubation, 3 μl samples were removed at different times, quenched with 20 μl loading buffer (50 mM Tris·HCl, pH 8, 10 mM EDTA, 5% SDS, 6% sucrose, 0.04% bromophenol blue, 0.04% xylene cyanole), and incubated with 1 μl proteinase K (Sigma Chem. Co.; 1 mg/ml) at room temperature for 2 h. DNA was then directly loaded and separated on 0.8% agarose gel, dried, and exposed.

Indirect Immunofluorescence Microscopy

Samples were first diluted 2× with ELB (250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 10 mM Hepes, pH 7.7) and then fixed by gently mixing with an equal volume of 3% formaldehyde and 2% sucrose in PBS for 10 min. After fixation, the nuclei were spun onto glass coverslips and treated with 0.1% Triton X-100 in PBS for 5 min. They were first stained with rabbit anti-RP-A antibody (1:100 in PBS), washed, and then stained with fluoresceine-labeled goat anti-rabbit antibody (Sigma Chem. Co.; 1:100 in PBS). Each staining was 1-h long and followed by three 5 min washes with PBS. Biotinylated dUTP incorporated into DNA was detected by streptavidin-Texas red conjugate (Sigma Chem. Co.; 1:100 in PBS) as the secondary antibody. Coil bodies were stained with a monoclonal mouse anti-fibrillarin antibody (generously provided by Dr. Joseph Gall) followed by goat anti-mouse Rhodamine conjugate (Sigma Chem. Co., 1:100 in PBS). Samples were finally mounted with Hoechst solution (0.01% in ELB) and viewed with a Zeiss microscope with epifluorescence. An integrating CCD camera (Colorado Video, Inc., Boulder, CO) was used for image collection and quantitation using the NIH Image software.

Other Methods

For Western blots, proteins were fractionated on 10% SDS-polyacrylamide gels, transferred onto PVDF membrane (Millipore Corp., Bedford, MA), and probed with the appropriate antibodies. ECL reagents (Amersham Corp., Chicago, IL) were used for detection. The Cipl protein was purified as a GST fusion from *Escherichia coli* transformed with an expression plasmid kindly provided by Dr. Steve Elledge (Harper et al., 1993).

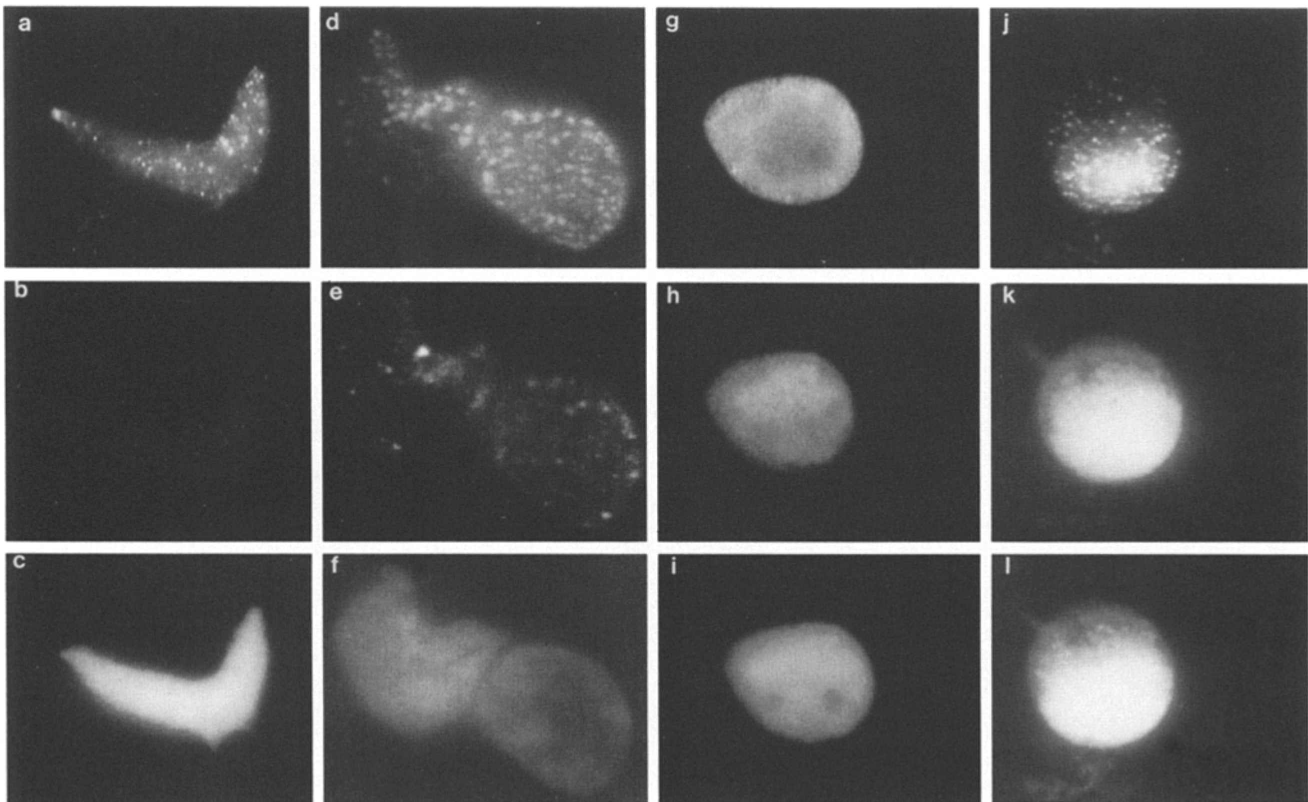

Figure 1. RP-A staining of sperm nuclei at different stages of replication. Demembranated sperm was mixed with cytosol, membrane, and biotinylated dUTP. At various times nuclei were fixed and stained for RP-A (*top*), biotin (*middle*), and DNA (*bottom*). (*a–c*) 15 min; (*d–f*) 30 min; (*g–i*) 60 min; (*j–l*) 150 min.

Results

RP-A Forms Foci on Sperm Chromatin both Before and After Replication

Crude extracts made from Xenopus eggs in the presence of the protein synthesis inhibitor cycloheximide are unable to synthesize mitotic cyclins A and B and, therefore, remain arrested in S-phase of the cell cycle (Murray and Kirschner, 1989). When demembranated sperm chromatin is added to such extracts, the chromatin serves as a template for nuclear formation (Lohka and Matsu, 1983) and all the DNA contained within these nuclei is replicated once (Blow and Laskey, 1986; Hutchison et al., 1987). These crude S-phase extracts can be separated via high speed centrifugation to generate several fractions, including a fraction containing the soluble cytoplasmic components and a fraction containing membrane components (Newport, 1987; Wilson and Newport, 1988). When these soluble and membrane fractions are combined, they reconstitute a mixture that will both form nuclei around demembranated sperm chromatin and allow this chromatin substrate to be replicated.

We have used this reconstituted cell-free system to examine the spatial distribution of the RP-A protein during DNA replication. To do this, demembranated sperm chromatin was incubated for different times in reconstituted extracts containing both soluble and membrane fractions and biotinylated dUTP. The resulting nuclei were fixed, pelleted onto glass coverslips, and then stained for RP-A and biotin-labeled dUTP (see Materials and Methods). The results from these experiments showed that during the early stages of nuclear formation RP-A associated with a large number of discrete foci on the chromatin (Fig. 1 *a*). The binding of RP-A to these chromatin sites preceded activation of DNA replication based on the absence of biotin staining at this stage (Fig. 1 *b*). At slightly later stages of nuclear formation, DNA replication initiated based on the appearance of punctate biotin staining (Fig. 1 *e*). Replication at this time colocalized with the RP-A foci has been previously reported (Fig. 1, *d* and *e*; Adachi and Laemmli, 1992). At later stages of replication both RP-A and replication staining uniformly distributed with DNA throughout the nuclei (Figs. 1, *g* and *h*). Quantitation of RP-A associated with DNA at this time showed that the total amount of RP-A bound to DNA had increased approximately 30-fold relative to nuclei displaying a foci staining pattern. These results suggest that RP-A is located at discrete organized centers before the initiation of replication and then distributes along single-stranded DNA at the replication fork following initiation of replication. The intense uniform staining pattern during replication would effectively obscure the staining of RP-A at organized centers if they persisted during this phase of replication. Therefore, based on this staining method, we can not determine whether the centers are stable during replication. However, following the completion of DNA replication (>150 min in extract), the RP-A associated with replicating DNA dissociates from the DNA. At this time we observe that punctate RP-A staining is again present within the nuclei (Fig. 1 *j*). This suggests that either the organized RP-A centers reform after the completion of DNA replication or that they are constantly present during the replication process. In a similar study it was reported that RP-A dissociated from the DNA following replication and accumulated within the lumen of the nuclei (Adachi and Laemmli, 1992). The presence of chromatin-bound RP-A foci following replication was not reported. We believe that this discrepancy is due to differences in immuno-

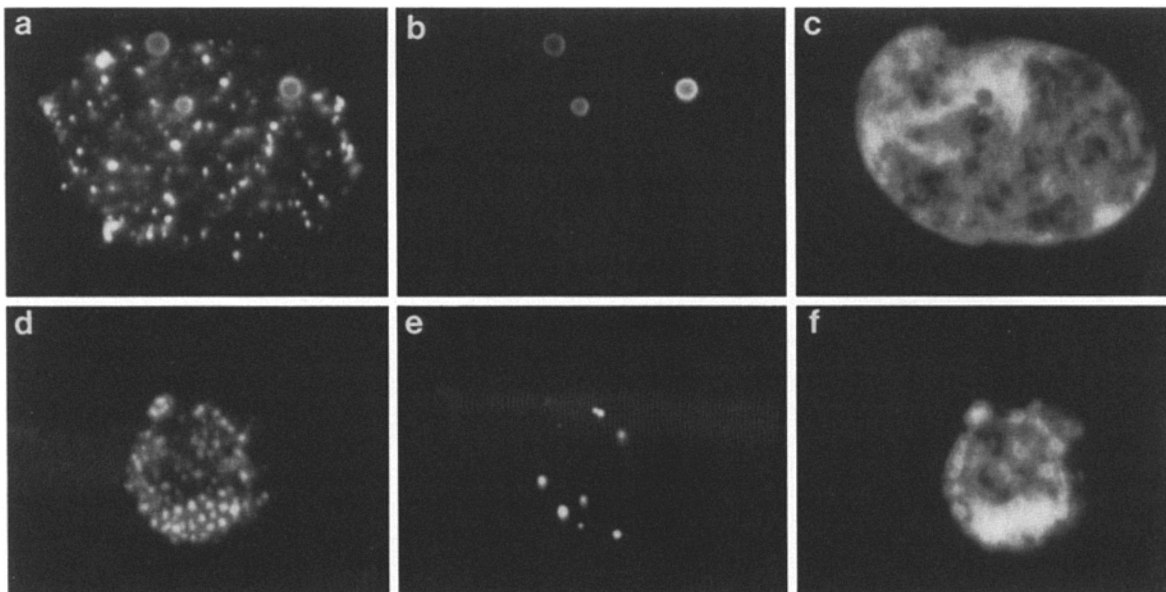

Figure 2. RP-A foci and coil body formation on lambda DNA. Lambda DNA and sperm were incubated with unfractionated interphase extracts for 60 min. The resulting nuclei were fixed and stained for RP-A (*a* and *d*), fibrillarin (*b* and *e*), and DNA (*c* and *f*). (*a–c*) Nuclei formed around sperm chromatin; (*d–f*) nuclei formed around lambda DNA. The weak signal of coil bodies in the RP-A fields (fluoresceine channel) is due to the leakage of light from the strong fibrillarin staining (Rhodamine channel). The number of coil bodies varies from 1 to 20 per nucleus and there appears to be an inverse relationship between their size and number.

staining methodology. In particular, when we pellet nuclei onto coverslips, RP-A unbound to DNA is released from the broken nuclei. As a result, this free RP-A does not mask the presence of organized RP-A foci following DNA replication.

Organized RP-A Foci Formation on Lambda DNA

The binding of RP-A to a limited number of discrete foci on sperm chromatin before the formation of nuclei and without DNA replication indicates that certain subdomains of the sperm chromatin are uniquely organized relative to the rest of the bulk chromatin. This unique organization could either pre-exist within the sperm chromatin before incubation in the extract or could be formed by proteins present in the extract. Moreover, because these foci appear to form only on a distinct subset of chromatin, it is important to understand the parameters that make this chromatin unique. With respect to this question one can propose two solutions. First, unique DNA sequences spaced relatively evenly along the DNA might serve as sites for the binding of specific proteins. Binding of these proteins to these specific sequences followed by aggregation of the proteins could act to generate a unique set of foci to which RP-A would bind. Alternatively, formation of these foci might occur in a sequence-independent process involving an exclusion mechanism. In this model, binding of foci-forming proteins to DNA would be sequence independent. However, once bound at one location, the protein would induce a change in chromatin structure to preclude its binding at adjacent DNA sequences. As in the first model, subsequent aggregation of these proteins could then generate foci. To determine whether RP-A foci formation is generated by the extract or results from a pre-existing organization of sperm chromatin and whether foci formation is dependent on specific DNA sequences, we have investigated how RP-A binds to bacteriophage lambda DNA added to Xenopus extracts.

When linear double-stranded bacteriophage lambda DNA is added to a mixture of soluble and membrane components derived from Xenopus eggs, the DNA is rapidly ligated into very large oligomeric units and then serves as a template for the assembly of nuclei which are morphologically indistinguishable from nuclei formed around sperm chromatin (Forbes, et al., 1983; Newport, 1987). Replication of the bacteriophage DNA occurs within nuclei (Newport, 1987), initiation appears to be sequence independent (Hyrien and Mechali 1992), and this replication localizes to discrete foci (Cox and Laskey, 1991). Using this initially protein-free DNA template, we have determined whether RP-A foci can form on the lambda DNA. To do this, lambda DNA was added to an unfractionated S-phase extract. After a 60-min incubation the nuclei that had formed around the ligated lambda DNA were fixed and stained for RP-A foci. As shown in Fig. 2 *d*, nuclei formed around lambda DNA, as nuclei formed around sperm chromatin, contained a large number of discrete foci containing RP-A. Labeling with biotinylated dUTP showed that the RP-A foci colocalize with early stages of DNA replication (data not shown). These results indicate that RP-A foci formation is the result of structural remodeling of DNA in the extract rather than the pre-existing structures on sperm chromatin before incubation in the extract. Moreover, because RP-A binding foci form on lambda DNA,

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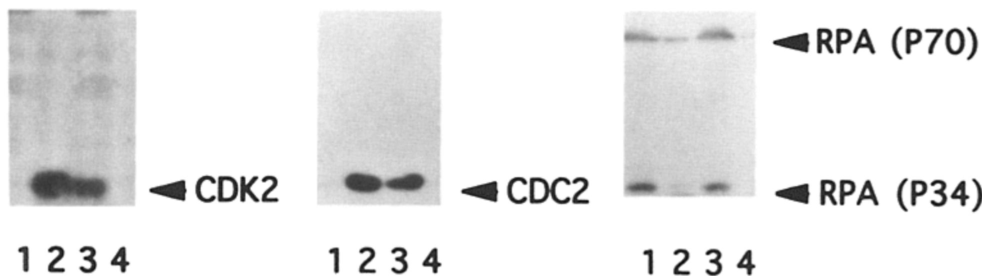

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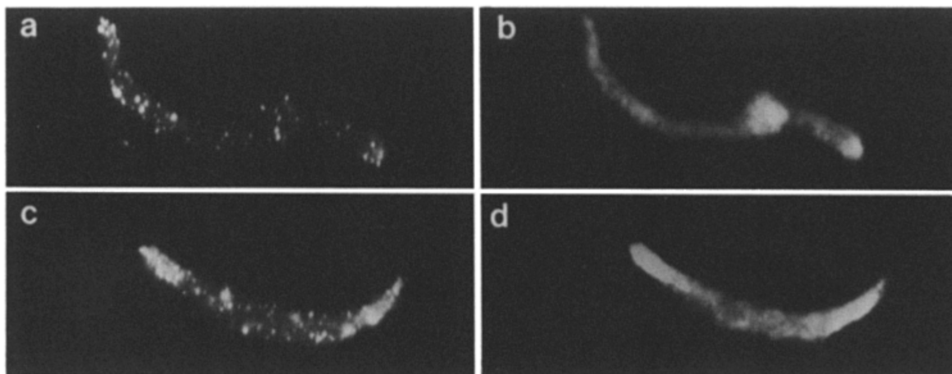

Figure 3. RP-A foci formation on decondensed sperm chromatin in the absence of cdk2. (*A*) p13 Sepharose beads or mock Sepharose beads were incubated with the soluble fraction of interphase egg extract and then removed. The beads and supernatants were adjusted to the same volume and subjected to Western analysis for cdk2 (*left*), cdc2 (*center*), and RP-A (*right*). (Lane *1*) p13-depleted supernatant; (lane *2*) p13 beads-bound fraction; (lane *3*) mock-depleted supernatant; (lane *4*) mock beads-bound fraction. (*B*) Demembranated sperm were incubated with the extract depleted with either p13 beads (*top*) or mock beads (*bottom*) in the absence of membrane. After 60 min, the decondensed sperm was fixed and stained for RP-A (*a* and *c*) and DNA (*b* and *d*).

this indicates that foci formation is independent of DNA sequences unique to the eukaryotic genome.

In Fig. 2 we also stained for coil bodies, a structure composed of many splicing proteins and small RNAs (for review see Wu and Gall, 1993). Two groups have recently reconstituted coil bodies in nuclei formed around either lambda DNA (Bell et al., 1992) or sperm chromatin (Wu and Gall, 1993) in Xenopus egg extracts. Because the morphology of these coil bodies is suspiciously similar to that of RP-A foci, it is possible that they might be the same entity. As shown in Fig. 2 (*b* and *e*), the coil bodies stained by anti–fibrillarin antibody are clearly distinct from RP-A foci (Fig. 2, *a* and *d*) in localization, size, and number. This is consistent with the putative roles for RP-A in DNA replication and coil bodies in RNA splicing.

Formation of RP-A Foci Is Not Dependent on cdk2 Kinase

Previously published results have demonstrated that replication of sperm chromatin in Xenopus egg extracts is dependent on the presence of a nuclear envelope surrounding the chromatin (Blow and Laskey, 1986; Newport, 1987; Sheehan et al., 1988). When sperm chromatin is incubated in the soluble fraction of an extract without membrane, DNA replication does not occur. Interestingly, Adachi and Laemmli (1992) have recently shown that the association of RP-A with sperm chromatin to form organized foci still occurs in the absence of membrane. This demonstrates that the organized binding of RP-A to chromatin foci can occur prior to the initiation of replication. Because cdk2 kinase has been implicated as playing an essential role in the early stages of the replication process, we have determined whether cdk2 is required for establishing these organized RP-A centers. To do this, the soluble fraction of an egg extract was depleted of cdk2 kinase by incubation with p13-Sepharose (similar results were obtained with anti–cdk2 antibody; data not shown). As shown in Fig. 3 *A*, cdk2 was quantitatively removed from the extract, while its level remained unchanged in the mock-depleted control extract. Following cdk2 depletion, demembranated sperm was added to the extract and incubated for 60 min before fixation and staining with anti–RP-A antibodies. The results from these experiments showed clearly that RP-A bound to organized chromatin foci as efficiently in cdk2-depleted extracts as it did in mock depleted control extracts (Fig. 3 *B*, compare *a* and *c*). This result demonstrates that cdk2 kinase is not required for the binding and assembly of RP-A to organized chromatin centers.

Previously we have demonstrated that cdk2 kinase is essential for replication of sperm chromatin templates added to Xenopus extracts (Fang and Newport, 1991). By contrast, the observation presented above indicates that cdk2 is not necessary for the formation of organized chromatin centers. Together these observations predict that the cdk2-depleted extract would form nuclei in which RP-A would associate with pre-replication centers but replication would be inhibited. To test this prediction, membrane, biotin-labeled dUTP, and sperm chromatin were added to the cdk2-depleted extract. Samples were removed at 60 and 90 min and stained for RP-A and biotin. At 60 min in cdk2-depleted extracts RP-A was observed to be bound to numerous (200–300) foci within the nuclei that formed around the sperm chromatin (Fig. 4 *a*). DNA replication at 60 min was completely absent in most nuclei. In a small fraction of nuclei, DNA replication was restricted to a few (10–20) foci that often co-localized with a subset of the RP-A foci (Fig. 4 *b*). Since we never saw

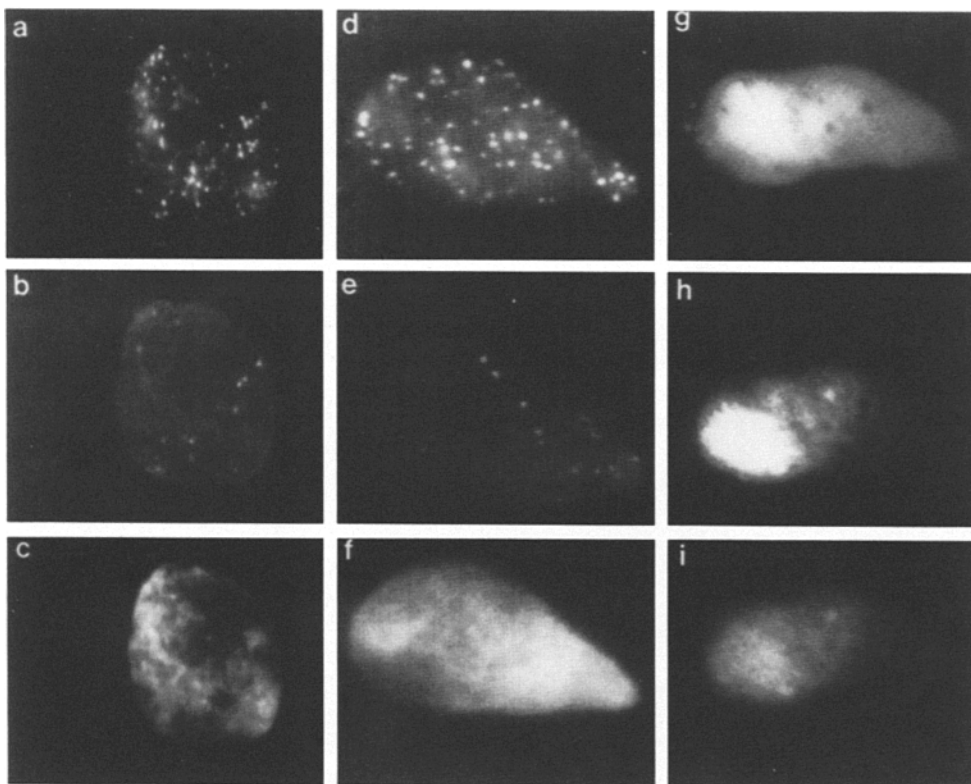

Figure 4. RP-A staining of nuclei formed in the absence of cdk2. Sperm was incubated with p13- or mock-depleted extract, membrane, and biotinylated dUTP for various times. The nuclei formed were fixed and stained for RP-A (*top*), biotin (*middle*), and DNA (*bottom*). (*a–c*) p13-depleted extract, 60 min; (*d–f*) p13-depleted extract, 90 min; (*g–i*) mock-depleted extract, 60 min. In the nuclei shown a small number of foci are labeled with biotin (*b* and *e*) and they usually colocalize with a subset of the RP-A foci (*a* and *d*). The size of foci in the nucleus at 90 min appears to be larger than those in the nucleus at 60 min, but this difference is not consistently observed.

any biotinylated dUTP incorporated into sperm chromatin in the absence of membrane (data not shown), these labeled foci most likely represent partial replication due to the residual activity of cdk2 (<5%). At 90 min RP-A still remained localized to a large number of foci and showed no indication of redistributing to DNA outside these foci (Fig. 4 *d*). Similarly, at 90 min very few of these foci were labeled with biotin, demonstrating that replication was effectively inhibited by cdk2 depletion (Fig. 4 *e*). Similar results were also obtained with nuclei formed around lambda DNA (data not shown). These observations demonstrate that in the absence of cdk2 stable RP-A binding centers can and do form within nuclei.

Increased Binding of RP-A to DNA Is Dependent on Initiation But Not Elongation

Above we have shown that in the absence of cdk2 kinase, initiation of DNA replication does not appear to occur and RP-A remains bound to organized foci but not to DNA located outside these foci. By contrast, in actively replicating nuclei, the total amount of RP-A bound to chromatin increases about 30-fold and this RP-A appears to be distributed over all the DNA in the nucleus. These observations suggest that large amounts of RP-A bind to DNA at the replication fork following initiation. Thus, the increased binding and redistribution of RP-A could be used as a sensitive indicator of initiation events in the absence of elongation. To test this possibility, we have examined the change in RP-A amount and distribution under conditions in which initiation of replication proceeds but elongation is inhibited.

Ara-CTP is a competitive inhibitor of the elongation process of replication. Based on incorporation of radioactively labeled dATP into DNA, we have determined that addition of 125 μM Ara-CTP to an egg extract decreases the rate of replication of sperm chromatin 30-fold relative to a control (data not shown). Using Ara-CTP we have determined how RP-A levels and distribution are affected when elongation is inhibited. To do this, sperm chromatin was incubated in a reconstituted egg extract containing biotin-dUTP and Ara-CTP. After a 60- and 90-min incubation, nuclei were fixed and stained for RP-A and DNA replication. As shown in Fig. 5 *a*, at 60 min in many nuclei, RP-A was located both at a large number of discrete foci and lightly distributed over bulk chromatin. The number of RP-A staining foci present at this time in the presence of Ara-CTP was approximately equal to the number of foci present in nuclei incubated in cdk2-depleted extracts (compare Figs. 4 *a* and 5 *a*). Yet in most nuclei, especially by 90 min, in the presence of Ara-CTP discrete RP-A foci were no longer visible within nuclei due to the significantly increased fluorescent staining of RP-A bound to bulk chromatin (Fig. 5 *d*). Using a linearly calibrated integrating CCD camera to quantitate the increase in fluorescence due to RP-A associated with chromatin, we have determined that the amount of RP-A bound to chromatin increases about 30-fold. This is similar to the increase in chromatin associated RP-A fluorescence observed in control extracts lacking Ara-CTP and occurs even though replication is inhibited 30-fold relative to such control extracts (data not shown; compare Figs. 1 *h* and 5 *e*). These results show that the increase in chromatin-associated RP-A fluorescence occurs after initiation of replication and is independent of the rate of elongation of the replication complex. Therefore, the observation that RP-A remains associated with foci and does not distribute to bulk chromatin in the absence of cdk2 strongly suggests that cdk2 kinase is required for a process which occurs after formation of pre-replication centers but before initiation of DNA replication.

Cip1 Protein Slows both Initiation and Elongation

The establishment of leading and lagging strand DNA replication appears to occur in at least two distinct stages (Waga and Stillman, 1994). In the first step, double-stranded DNA is opened and polymerase α synthesizes a 100-200-bp primer on the leading strand. Following this priming step, the second stage of replication involves the coordinated elongation of both leading and lagging strands. The activation of this second stage of replication is dependent on the polymerase δ accessory protein PCNA. We have shown that depletion of cdk2 from an extract strongly inhibits the incorporation of biotin-labeled dUTP into DNA within nuclei (Figs. 4, *b* and *e*). Moreover, under these conditions RP-A remains localized to discrete foci and does not bind to the bulk of the DNA. Together these observations suggest that cdk2 is required for the priming stage of replication. However, because the priming reaction results in the addition of only 100–200 bp of DNA, it is possible that the priming reaction has occurred in the absence of cdk2 but the biotin signal generated is below the limit of detection of our methods. To address this issue, we have used rate-limiting amounts of the Cip1 protein to slow down the replication process during the initiation stage.

Cip1 is an inhibitor of cdk2 kinases in both mammalian cells (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993) and Xenopus eggs (Guadagno and Newport, unpublished result). More recently, using the SV-40 replication system, it has been shown that Cip1 also binds to PCNA in a cdk2-independent manner and that the association of Cip1 with PCNA inhibits the post-priming stage of the replication reaction (Waga et al., 1994). Following these initiation stages Cip1 does not appear to inhibit further elongation (Strausfeld et al., 1993). Therefore, if cdk2 kinase activity is required for the initial priming stage of replication, we would expect that in the presence of Cip1 both the priming reaction (cdk2 sensitive) and further elongation (PCNA sensitive) would be significantly delayed. Alternatively, if cdk2 was only necessary for the post-priming reaction, then Cip1 would not delay the priming stage but would significantly delay further elongation by inhibiting both cdk2 and PCNA. In this case, we would expect to observe little delay in the accumulation of primed replication complexes.

To distinguish between these two possibilities, either a glutathione S transferase (GST)-Cip1 fusion protein or GST alone was added to extracts containing soluble components and incubated for 20 min. Following this, sperm chromatin, membrane, and biotin-labeled dUTP were added to the reactions. After a further 60 and 120 min, aliquots of the reactions were fixed and stained for RP-A and biotinylated dUTP incorporated into DNA. At 60 min in control reactions containing GST alone, extensive DNA replication had occurred as indicated by the co-localization of biotinylated-dUTP with all the DNA within nuclei (Fig. 6, *h* and *i*). Similarly, at this time in control extracts, RP-A staining co-localized with bulk DNA and discrete foci were no longer discernible (Fig. 6 *g*). In contrast, at 60 min in extracts treated with GST-Cip1

protein, biotin incorporation into DNA was negligible and RP-A was located exclusively at foci (Figs. 6, *a* and *b*). However, after a 120-min incubation in the presence of Cipl, RP-A staining increased about 10-fold and now co-localized with total DNA (Fig. 6 *d*). Importantly, at this time biotin incorporation into DNA appeared to be restricted to a large number of discrete foci (Fig. 6 *e*). Using a CCD camera to capture the biotin image and calculate the average intensity per total nuclear area, or by directly measuring ^{32}P-dCTP incorporation into chromatin, we estimate that in the presence of Cipl the total replication at 120 min is less than 1% of the replication which has occurred in control nuclei. That these biotin-labeled foci do not occur in the absence of cdk2 (i.e., cdk2-depleted extracts) and appear only after an extended delay (120 min) in the presence of Cipl supports the conclusion that cdk2 is essential for the first step of replication initiation. Furthermore, they suggest that once replication passes through the cdk2-dependent step, it can be specifically arrested again following synthesis of a primer, presumably due to the inhibition of PCNA activity by Cipl (Waga et al., 1994).

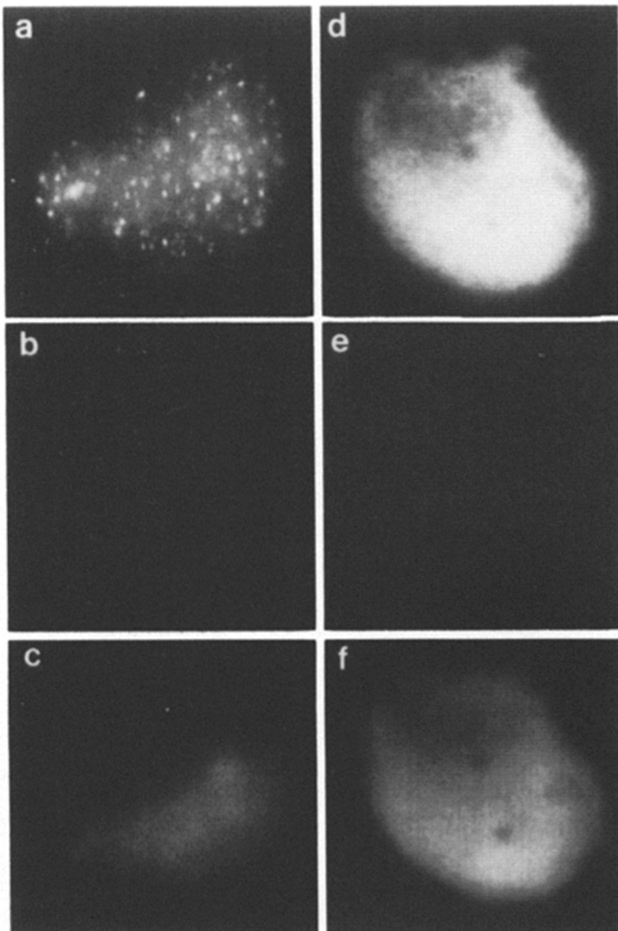

Figure 5. RP-A staining when replication is inhibited by Ara-CTP. Sperm nuclei were reformed in the presence of 10 μM biotinylated dUTP and 125 μM Ara-CTP. After 60 min (*a–c*) or 90 min (*d–f*), nuclei were fixed and stained for RP-A (*top*), biotin (*middle*), and DNA (*bottom*).

Depletion of cdk2 during Mitosis Does Not Prevent Subsequent Foci Formation during S-Phase

In the experiments described above, we have shown that depletion or inactivation of cdk2 kinase from an extract already in S-phase completely blocks DNA replication but does not inhibit RP-A foci formation. Recently, two reports (Blow, 1993; Kubota and Takisawa, 1993) have shown that addition of kinase inhibitors (6-DMAP or Staurosporin) to mitotic egg extracts blocks DNA replication of sperm chromatin when these treated extracts are induced to enter S-phase. Interestingly, the inhibition of replication was only observed when the inhibitors were added either during mitosis or immediately following activation of the extract (Blow, 1993). These results suggest that a kinase may function either during or immediately after mitosis to potentiate DNA replication during S-phase. In egg extracts, cdk2 kinase is active during both S-phase and mitosis (Fang and Newport, 1991). Therefore, it is possible that mitotically active cdk2 kinase is required for formation of RP-A foci.

To test this possibility, we depleted cdk2 activity from extracts arrested in mitosis (CSF extracts; see Materials and Methods). Following depletion, the extracts were induced to enter S-phase by addition of Ca^{+}. When sperm chromatin was added to these pre-treated S-phase extracts, they formed nuclei, although the nuclei were smaller than those of the control. As expected, they failed to replicate based on the absence of biotin labeling (Fig. 7 *b*). However, when these nuclei were stained with anti–RP-A antibody it was clear that RP-A was still bound at discrete foci (Fig. 7 *a*). These results indicate that the association of RP-A with foci is independent of the cdk2 activity present during either mitosis or S-phase.

Addition of 6-DMAP at Mitosis Does Not Inhibit Replication Initiation but Does Block Elongation

As mentioned above, treatment of mitotic extracts with 6-DMAP blocks subsequent DNA replication when the extract is activated. However, addition of this inhibitor to an extract that is already in S-phase does not inhibit DNA replication (Blow, 1993). By contrast, we have shown that DNA replication is inhibited when cdk2 is depleted from either mitotic or S-phase extracts. Together these observations suggest that cdk2 is not the 6-DMAP–sensitive kinase required during mitosis to potentiate subsequent DNA replication. Because association of RP-A with organized foci might be part of the kinase-dependent potentiation process, we have determined how addition of 6-DMAP to extracts affects RP-A foci formation. To do this, 6-DMAP was added to mitotically arrested extract, which was then activated by addition of Ca^{+}. Sperm chromatin and biotinylated dUTP were then added. Aliquots of this reaction were removed at 15 and 60 min and stained for RP-A and DNA replication. Early during the incubation (15 min), RP-A staining was localized to discrete foci and little DNA replication was observed (Fig. 8, *a* and *b*). This result demonstrates that the association of RP-A with foci is insensitive to 6-DMAP. As such the formation of these foci does not depend on the activity of kinases inhibited by this drug. Quite surprisingly, by 60 min the staining pattern of both RP-A and biotin had changed significantly (Fig. 8, *d* and *e*). The total amount of RP-A bound to chromatin had increased significantly to about 30% of that found in controls at the same time. Moreover, this RP-A was

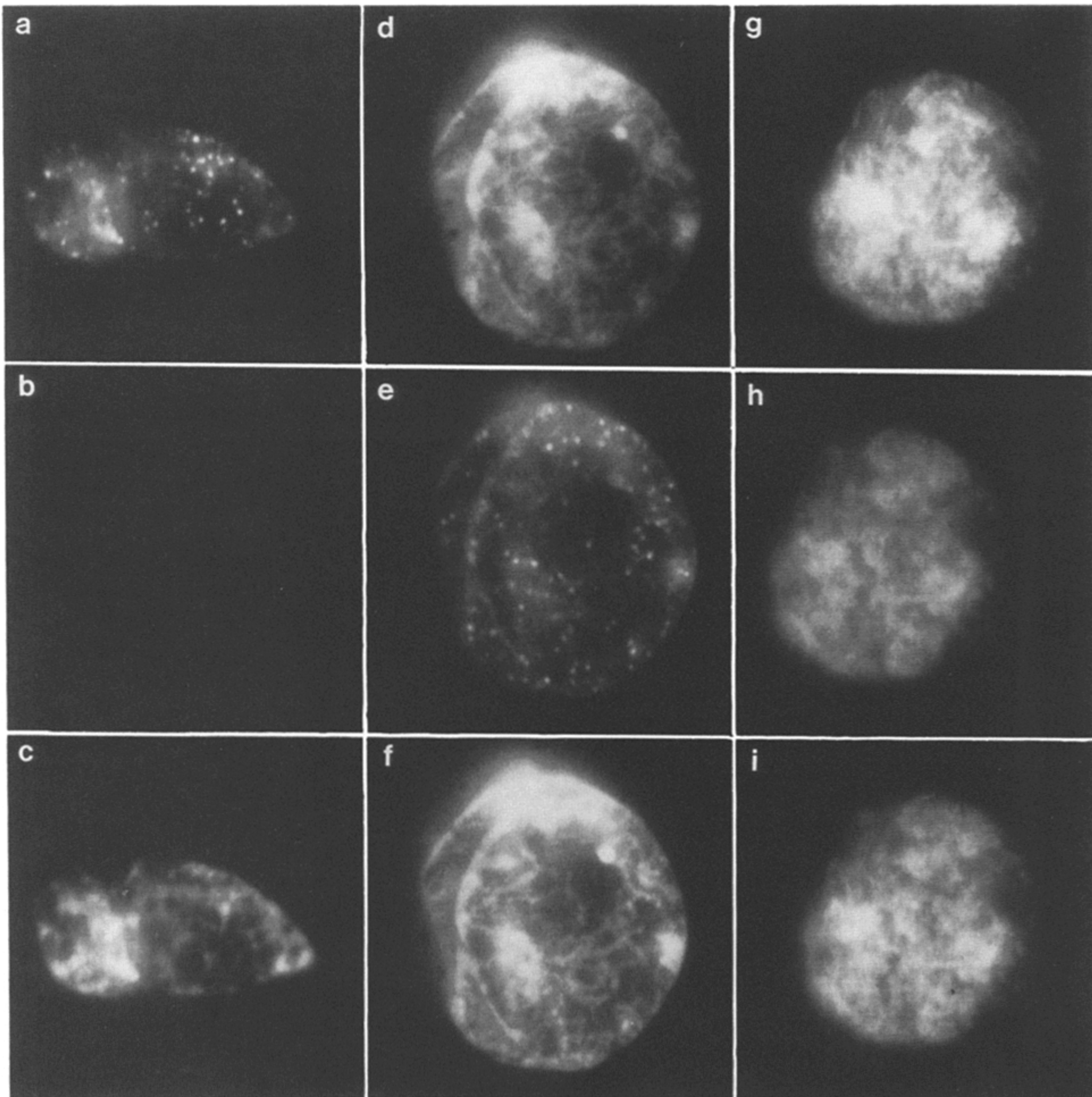

Figure 6. The effect of Cipl on RP-A foci formation and replication. The soluble fraction of an interphase extract was pre-incubated with 300 nM GST-Cipl or GST alone for 20 min. Sperm, membrane, and biotinylated dUTP were then added and incubated for various times. The nuclei formed were then fixed and stained for RP-A (*top*), biotin (*middle*), and DNA (*bottom*). (*a–c*) +Cipl, 60 min; (*d–f*): +Cipl, 120 min; (*g–i*) +GST, 60 min.

distributed over all the DNA and discrete foci were no longer visible (Fig. 8 *d*). In the experiments described above, we have shown that this increase in total chromatin associated RP-A and its redistribution to bulk chromatin indicates that the first step in initiation of DNA replication has occurred. Consistent with this, we observed significant biotin incorporation into DNA at 60 min. Importantly, like Cipl-treated extracts, this biotin incorporation was confined to discrete foci and unlike RP-A, did not extend into the surrounding bulk chromatin. This foci-associated staining pattern of DNA replication persisted with further incubation and was unchanged by 90 min (data not shown). We estimate based on quantitative analysis of CCD camera images that the total replication which has occurred within all the foci of a nucleus is less than 1% of the replication which has occurred in control nuclei at 60 min. Taken together our results demonstrate several points. First, our observations confirm the published results that pre-treatment of mitotic extracts with 6-DMAP causes a significant inhibition in DNA replication when the extracts are subsequently activated to enter S-phase (Blow, 1993). Second, we have demonstrated that the observed inhibition of DNA replication in pre-treated extracts is not due to the absence of RP-A binding to organized foci or replication centers. Third, our finding that DNA replica-

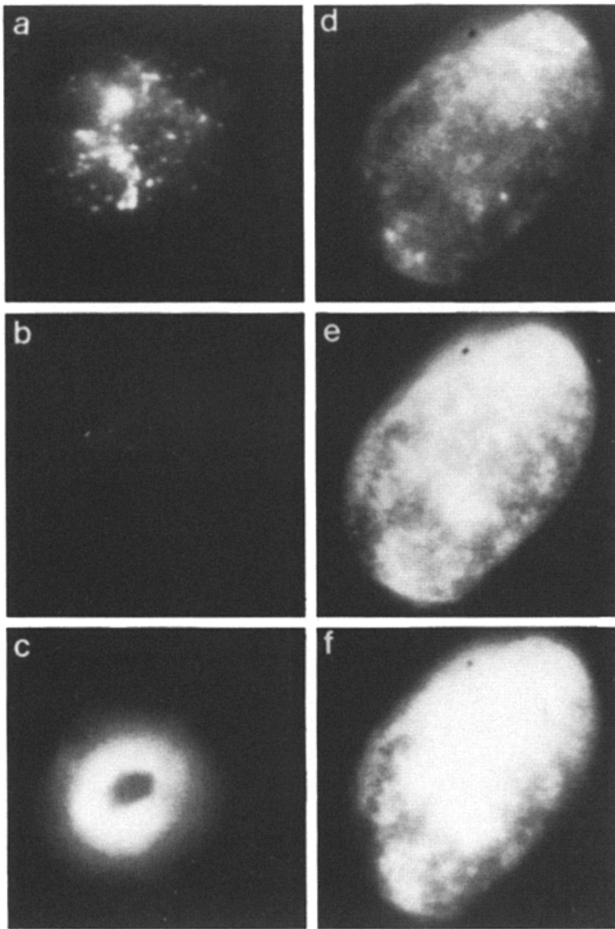

Figure 7. RP-A formation when cdk2 is depleted from mitotic extracts. CSF extracts were depleted with p13 beads or mock beads. Immediately after the extracts were activated with 0.4 mM $CaCl_2$, sperm, membrane, and biotinylated dUTP were added. Nuclei were stained 60 min later for RP-A (*top*), biotin (*middle*), and DNA (*bottom*). (*a–c*) nucleus formed in the p13-depleted extract; (*d–f*) nucleus formed in the mock-depleted extract.

tion in pre-treated extracts is confined to discrete foci while RP-A staining occurs generally along all DNA suggests that 6-DMAP treatment does not limit initiation of DNA replication and synthesis of a DNA primer. Rather, our results indicate that the primary defect caused by 6-DMAP pre-treatment is to block a step that converts replication from the priming mode to the elongation mode for the continuous replication of both leading and lagging strands.

M13 Single-Stranded DNA Replication Is Dependent on RP-A but Not Inhibited by Cip1

Both Cip1 and 6-DMAP can arrest replication at a stage where biotin-labeled DNA remains tightly confined to foci while RP-A binding increases greatly (~10-fold) and colocalizes with total DNA. A possible explanation for this result would be that once replication has initiated and a primer is synthesized, RP-A in conjunction with a helicase can continue to generate and bind to regions of single-stranded DNA well in front of the stalled replication complex. Measurements of the distances between foci show that most foci are separated from each other by 1-3 μm. Therefore, assuming a base-pair separation distance of 3.5 Å, we calculate that the DNA located between foci is 3,000–9,000 base pairs long. This is undoubtedly a very conservative underestimate because it ignores the compaction of DNA due to association with nucleosomes and other folding arrangements. Nonetheless, even using this conservative estimate, if single-stranded DNA is generated ahead of stalled replication forks, then the uniform RP-A staining pattern that we observe suggests that these regions would be at least 3000 to 9000 base pairs in length. Such long stretches of single-stranded DNA not used as template for DNA replication is at odds with published results showing that single-stranded DNA is rapidly and efficiently replicated in extracts depleted of cdk2 or pretreated with 6-DMAP (Blow and Nurse, 1991; Blow, 1993). In an attempt to resolve this apparent contradiction, we tested both whether RP-A is necessary for replication of single-stranded DNA added to S-phase extracts and whether addition of Cip1 to such extracts blocks replication of single-stranded DNA.

To determine whether RP-A is necessary for replication of single-stranded DNA, RP-A antibody was bound to protein A–Sepharose beads and these beads were then used to deplete an extract of RP-A. Previously, we have shown that this procedure removes over 95% of the RP-A from an extract and results in a complete inhibition of DNA replication using sperm chromatin as template (Fang and Newport, 1993). To determine if replication of single-stranded DNA is similarly inhibited by RP-A depletion, we have added M13 single-stranded DNA to an RP-A–depleted extract and measured the incorporation of radioactively labeled dCTP into this template. The results from such experiments clearly show that RP-A–depleted extracts are unable to replicate the template well while mock-depleted control extracts replicate this single-stranded DNA template efficiently (Fig. 9, *two left panels*). Therefore, RP-A appears to be required for the replication of single-stranded DNA added to egg extracts.

We next asked whether adding Cip1 to extracts blocked replication of single-stranded DNA. As shown in Fig. 9, addition of Cip1 to extracts does not inhibit replication of single-stranded DNA templates (*two right panels*). Together these results indicate that if, as we proposed at the beginning of this section, RP-A binds to long regions of DNA in front of Cip1-arrested replication complexes, then this single-stranded DNA should be replicated efficiently. This replication would in turn effectively obscure the punctate biotin replication pattern. That this does not occur may indicate that long stretches of single-stranded DNA are not generated in front of the replication complex stalled by Cip1 or 6-DMAP treatment. An alternative explanation for this observation is presented in the discussion section.

Discussion

How Are RP-A Foci Organized and Regulated

Using an in vitro system derived from Xenopus eggs, we have investigated several aspects of RP-A foci organization (Fig. 1 *a*; Adachi and Laemmli, 1992). With respect to the structural requirement of these foci, we have shown that they originate as a result of DNA rearrangements induced by factors present in the Xenopus extracts and are not a preexisting condition present on sperm chromatin. In addition,

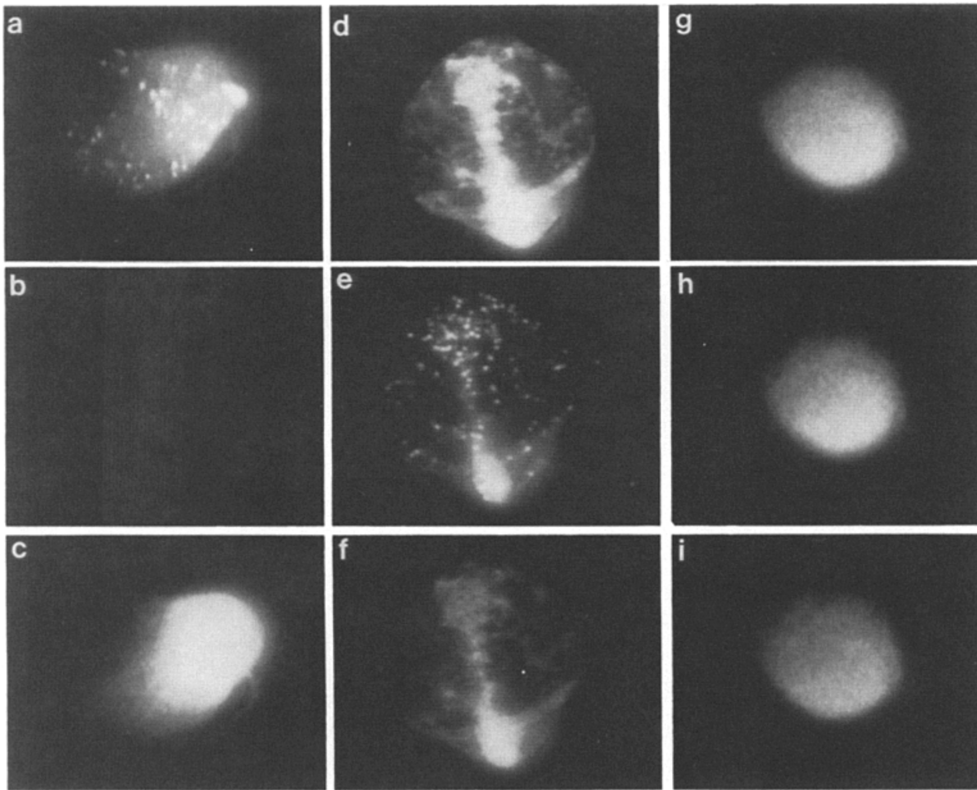

Figure 8. The effect of 6-DMAP on RP-A staining and DNA replication. CSF extracts were activated with $CaCl_2$ in the presence or absence of 3 mM 6-DMAP (Sigma Chem. Co.). Sperm, membrane, and biotinylated dUTP were then added and incubated for various times. Nuclei were fixed and stained for RP-A (*top*), biotin (*middle*), and DNA (*bottom*). (*a–c*) + 6-DMAP at 15 min; (*d–f*) + 6-DMAP at 60 min; (*g–i*) no 6-DMAP at 60 min.

RP-A foci formation does not appear to require specific eukaryotic DNA sequences. These conclusions are based on the observations that RP-A forms foci in nuclei formed around protein-free bacteriophage lambda DNA and these RP-A foci colocalize with DNA replication (Fig. 2; unpublished results; Cox and Laskey, 1991).

With respect to the regulation of RP-A foci formation, we find that the association of RP-A with organized clusters is independent of cdk2 kinase activity. In support of this conclusion, we have shown that RP-A associates with chromatin foci even when cdk2 is depleted by p13 (Figs. 3, 4, and 7) or inhibited by Cipl (Fig. 6). Following completion of this study, Adachi and Laemmli published a paper arriving at a similar conclusion when extracts were depleted with p13 beads or Cipl beads (1994). We have also shown that the association of RP-A with foci appears to be independent of all the kinases sensitive to the general kinase inhibitor 6-DMAP (Fig. 8). However, we do not see RP-A association with chromosomes in mitosis (unpublished result), suggesting that the mitotic kinase cdc2-cyclin B may negatively regulate this association. Consistent with this, we have shown that RP-A in Xenopus extracts is heavily phosphorylated by cdc2 at mitosis and then rapidly dephosphorylated as the extract leaves mitosis (Fang and Newport, 1993). Thus, phosphorylation and dephosphorylation of RP-A by cdc2 may be part of the regulatory process that controls the dissociation of RP-A from foci at the beginning of mitosis and its subsequent reassociation at the end of mitosis.

Regarding the persistence of these organized clusters, we find that during DNA replication RP-A staining is uniform along all DNA. However, we observe that discrete RP-A foci reappear on chromatin following the completion of replication (Fig. 1 *j*). This suggests that either these foci are maintained during replication or proteins interact with chromatin to form new foci following replication. These results in combination with the observation that RP-A foci can form on chromatin before nuclear formation (Fig. 1 *a*; Adachi and Laemmli, 1992) suggest that the underlying proteins responsible for clustering replication loops together into foci may be associated with chromatin throughout cell cycle except in mitosis. This is clearly consistent with the recent demonstra-

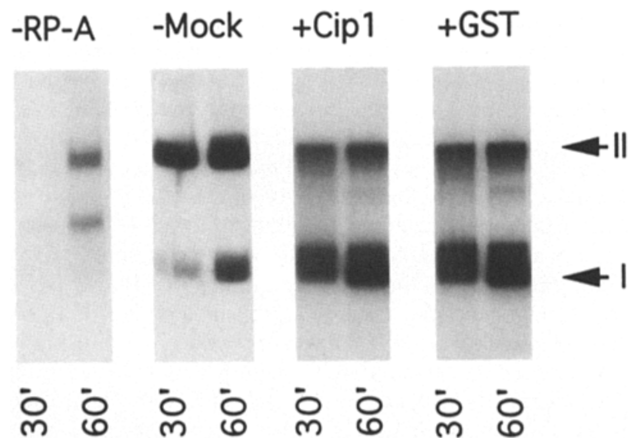

Figure 9. M13 single-stranded DNA replication. M13 DNA (0.25 ng/μl) was replicated in soluble interphase fractions depleted of RP-A or pre-treated with 300 nM Cipl or GST for 30 or 60 min. ^{32}P-dCTP was included in the reactions to follow the extent of replication. The RP-A depletion experiment and Cipl treatment experiment were done on separate occasions and, as a result of slightly different processing procedures, have different ratios of the supercoiled form (*I*) and relaxed form (*II*).

tion that the ORC in yeast is constantly associated with origin sequences throughout interphase (Diffley et al., 1994).

Does Replication Occur Exclusively in Association with Foci

With regard to the functional relationship between DNA replication and foci formation, a large number of labeling experiments using modified nucleotides that can be detected following their incorporation into DNA have shown that DNA replication can and does initiate at discrete foci within nuclei (Nakamura et al., 1986; Nakayasu and Berezney, 1989; Mills et al., 1989; Cox and Laskey, 1991). However, from these experiments it can not be concluded that replication initiates exclusively at these foci. The results from two separate experiments presented in this report force us to consider this issue. Specifically, when cdk2 is depleted from an extract, DNA replication is completely inhibited and RP-A remains tightly associated with discrete foci (Fig. 4). By contrast, under certain conditions Cipl and 6-DMAP can inhibit replication at the elongation but not the priming stage (Figs. 6 and 8). Interestingly, although newly replicated primer DNA remains confined to discrete foci within the nucleus, RP-A fluorescence increases 10-fold and co-localizes with all the DNA in the nucleus. Thus, under these conditions it would appear that RP-A binding does not co-localize with DNA replication. We have considered two explanations for this data.

One explanation is that RP-A in combination with a helicase continues to generate and bind to single-stranded DNA even when the replication complex is stalled following primer synthesis. This seems unlikely for two reasons. First, the distances between adjacent foci are greater than 3 μm in many cases. In the most conservative estimate assuming no compaction due to association with histones, this would result in 9,000 base pairs of single-stranded DNA in front of the stalled replication complex. However, studies based on SV-40 replication suggest that there is very limited amount of DNA unwinding in front of a stalled replication fork (Droge et al., 1985). Moreover, if they existed they would be replicated based on the observation that replication of M13 single-stranded DNA is dependent on RP-A but not inhibited by Cipl or 6-DMAP (Fig. 9; Blow, 1993). A second argument can be made based on the observation that the total amount of RP-A bound to DNA in the presence of Cipl or 6-DMAP is actually less (~30%) than that in actively replicating nuclei. This is the opposite of what would be expected if RP-A continued to generate single-stranded DNA in advance of the replication fork. Together these observations strongly suggest that the extensive binding of RP-A to DNA away from foci is not due to significant melting of DNA ahead of the stalled replication complex.

An alternative explanation for the differential localization of RP-A and DNA replication in Cipl- or 6-DMAP–arrested nuclei is to assume that DNA replication can initiate at sites other than foci. For example, it has been estimated that foci are composed of 300–1,000 origins clustered together (Mills et al., 1989). Therefore, if each of these origins serves as a site for synthesis of a primer, the biotin fluorescent signal generated at foci would be 300–1,000 times brighter than the signal generated from a single origin site. Currently, we have to integrate the fluorescent signal for relatively long times before biotin-labeled foci are visible. As such, if single origin sites are randomly distributed within nuclei the diffuse nature of these sites would generate fluorescent signals that would be below our limits of detection. By contrast, because the signal generated by RP-A is much brighter than the biotin signal, we would expect that binding of RP-A to multiple unwound single origin sites randomly distributed along DNA would generate a visible fluorescent RP-A signal co-localizing with bulk DNA. These predictions are consistent with our observations and may indicate that origin usage is not confined to DNA associated with foci. Although the extreme case of priming at single origins is presented, it should be noted that the same arguments would apply if clusters of origins significantly less than 300–1,000 could aggregate to form foci. In this case, origin usage may still be restricted exclusively to foci, but the functional sizes of foci may vary greatly.

Which Step of DNA Replication Is Controlled by cdk2

Previously, we have demonstrated that cdk2 is essential to DNA replication of nuclei formed around sperm chromatin (Fang and Newport, 1991). In this report, we use the spatial distribution of RP-A and the incorporation of biotin-labeled dUTP into DNA as two markers to define the step at which cdk2 controls DNA replication. We find that when cdk2 is depleted from extracts, RP-A remains associated with foci and biotin dUTP incorporation into DNA is completely inhibited (Fig. 4). Similarly, in the presence of the Cipl protein we observe an initial delay in both RP-A binding and DNA synthesis (Fig. 6, *a* and *b*), consistent with the inhibition of cdk2 kinase activity by Cipl. The inhibition of cdk2 activity by adding Cipl to the extract is apparently not as tight as depleting it because following the initial delay chromatin bound RP-A increases dramatically and foci-localized replication occurs but elongation does not (Figs. 6, *d* and *e*). This second arrest is consistent with the direct inhibition of PCNA by Cipl (Waga et al., 1994). Together these results suggest that cdk2 functions after the formation of RP-A foci but before the synthesis of DNA primers.

There are two potential targets for cdk2 within this narrow window: origin unwinding and primer synthesis. Three lines of evidence suggest to us that it is the origin unwinding step which is controlled by cdk2. First, M13 single-stranded DNA replication, which is also dependent on priming (Mechali and Harland, 1982), is not affected by depletion of cdk2 or addition of Cipl (Fig. 9; Blow and Nurse, 1990). Second, we find that when DNA replication is arrested at the time of primer synthesis by Ara-CTP, the amount of RP-A bound to chromatin increases dramatically and it distributes uniformly with DNA (Fig. 5). This is in contrast to the foci RP-A staining pattern we observed when cdk2 is depleted, suggesting that cdk2 functions at a step earlier than primer synthesis. Third, we have shown that RP-A is phosphorylated during S-phase and this phosphorylation is dependent on the presence of single-stranded DNA (Fotedar and Roberts, 1992; Fang and Newport, 1993). When DNA polymerase α is inhibited by aphidicolin, RP-A is still phosphorylated, consistent with its binding to the single-stranded regions at the unwound origins. In contrast, cdk2-depleted nuclei, RP-A remains in the unphosphorylated state, suggesting that it is not associated with single-stranded DNA (Fang and New-

port, 1993). Together these observations suggest that the kinase activity of cdk2 is probably required for the origin unwinding step of DNA replication.

6-DMAP Inhibits the Post-Priming Polymerase Switching Step of Replication

Previously published reports have demonstrated that addition of the kinase inhibitors 6-DMAP or Staurosporin to extracts arrested in mitosis causes a significant subsequent inhibition of DNA replication when these extracts are allowed to enter S-phase (Blow, 1993; Kubota and Takisawa, 1993). Moreover, it has been shown that the sensitivity of DNA replication to 6-MAP is restricted to a very limited period of time following release of extracts from mitosis. If 6-DMAP is added into extracts already in interphase, replication occurs at control levels (Blow, 1993). These observations are consistent with the proposal that a kinase is required immediately following exit from mitosis to activate a licensing factor necessary for subsequent DNA replication (Blow, 1993; Kubota and Takisawa, 1993). Once activated by the kinase, the licensing factor is apparently stable, explaining why DNA replication becomes insensitive to 6-DMAP addition shortly after exit from mitosis. The observation that RP-A binds to discrete foci on sperm chromatin before nuclear envelope formation suggests that this RP-A binding might be a licensing event. To address this possibility, we have treated mitotic extracts with 6-DMAP, released them into S-phase and determined whether RP-A binds to foci under these conditions. Our results demonstrate that pre-treatment of mitotic extracts with 6-DMAP does inhibit DNA replication, but does not block the association of RP-A with foci (Fig. 8 *a*). Based on this data, it does not appear that RP-A binding to foci is a limiting step inhibited by 6-DMAP.

The reported inhibition of DNA replication in extracts pre-treated with 6-DMAP was primarily measured by monitoring incorporation of radioactively labeled nucleotide into sperm chromatin added to the extracts. Based on the significant inhibition observed (>95%), it was concluded that 6-DMAP treatment blocked replication by preventing initiation of DNA replication (Blow, 1993). However, because the vast majority (>99%) of the radioactive label incorporated into DNA by this method occurs during the elongation phase of replication, it is a relatively insensitive technique for determining whether initiation events have been inhibited. Using an integrating CCD camera and following biotin dUTP incorporation into DNA is a far more sensitive technique appropriate for analyzing initiation events. We have used this approach to carry out a detailed evaluation of which step in DNA replication is inhibited by 6-DMAP treatment. Our results show that biotin-label is rapidly incorporated into discrete foci within nuclei incubated by extracts pre-treated with 6-DMAP (Fig. 8 *e*). However, further DNA synthesis beyond this step is inhibited even after long incubations. Moreover, when these nuclei are stained for RP-A we find that RP-A staining co-localizes over all DNA and is present at levels ~30% of that observed in control nuclei that are actively replicating. Together these observations indicate that pre-treatment of extracts with 6-DMAP does not significantly affect origin opening and synthesis of a DNA primer but does effectively inhibit elongation of this primer.

Because it has been suggested that the 6-DMAP-sensitive factor is licensing factor (Blow, 1993), it is important to consider why this factor might be functioning late in the initiation process. One possible explanation for this is that origin opening and assembly of a stable replication complex is a multi-step process requiring the coordinated association of many proteins with each other. The successful synthesis of a primer strand represents the first molecular event indicating that this association has generated a productive replication complex which is fully functional for further DNA replication. Therefore, if a license was used to allow replication to proceed beyond this point, it would ensure that an origin had both been activated and activated productively. Since it is unlikely that an origin can be fired again to initiate primer synthesis in the same cell cycle, mechanisms in addition to this 6-DMAP sensitive factor might also be involved to keep replication to exactly one round per cell cycle.

Model

In Fig. 10 we present a model outlining a sequence of events for controlling initiation of DNA replication based on the results in this report as well as results published by other laboratories. Based on the model RP-A rapidly associates with chromatin foci, probably through protein-protein interaction with origin recognition factors, as the extract exits mitosis. The association of RP-A with foci at this time occurs before formation of the nuclear envelope and is unlikely to be a major control point based on the observation that it is independent of cdk2 kinase and kinases inhibited by 6-DMAP (Adachi and Laemmli, 1994; this report). During this same period, a 6-DMAP-sensitive kinase is required to activate a "licensing factor" needed for DNA replication (Blow, 1993; Kubota and Takisawa, 1993). Once activated, the licensing factor appears to be stable and inhibition of the kinase does not block subsequent DNA synthesis. Like

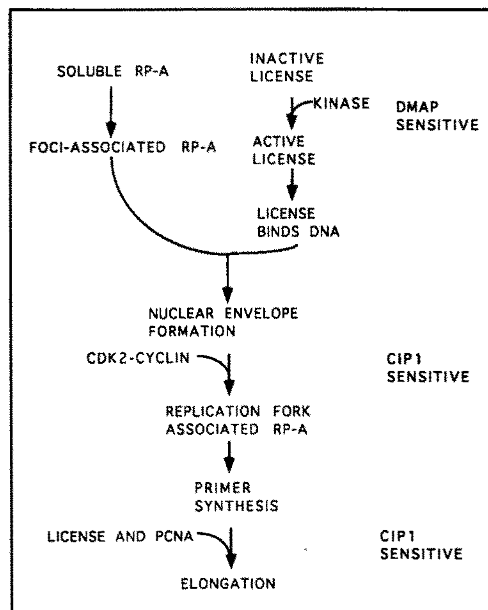

Figure 10. Summary of the potential cell cycle-regulated events that control eukaryotic DNA replication.

RP-A, the licensing factor appears to bind to DNA prior to the formation of the nuclear envelope. Subsequent to this binding, the formation of a nuclear envelope around chromatin is necessary for both excluding unbound licensing factor (Blow, 1993) and generating a structural environment needed for replication (Newport, 1987; Sheehan et al., 1988). Following these events, cdk2 kinase may activate a DNA helicase to unwind replication origins (Erdile et al., 1991), which then serves as a substrate for primer synthesis by DNA polymerase α-primase complex. As a result of this priming step, significant amounts of RP-A now bind to the newly exposed single-stranded DNA and these RP-A are phosphorylated by a single-stranded DNA dependent kinase (Fotedar and Roberts, 1992; Fang and Newport, 1993; this report). Finally a PCNA-dependent strand switching event occurs in which DNA polymerase δ replaces α for further coordinated elongation of both the leading and lagging strands (Waga and Stillman, 1994). Based on the observation that chromatin incubated in extracts pre-treated with 6-DMAP rapidly synthesizes a primer and binds RP-A extensively, we propose that the "6-DMAP sensitive licensing factor" is not required for the initiation phase of DNA replication. Rather, our data indicate that it is a component, like PCNA, necessary for forming a replication complex capable of carrying out coordinated leading and lagging strand synthesis. Our results also suggest that the Cipl protein could potentially exert cell cycle control over DNA replication at two points: before priming by inhibiting cdk2 and at strand switching by inhibiting PCNA (Waga et al., 1994; this report).

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