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Initiation of Eukaryotic DNA Replication: Origin Unwinding and Sequential Chromatin Association of Cdc45, RPA, and DNA Polymerase α

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Summary

We report that a plasmid replicating in *Xenopus* egg extracts becomes negatively supercoiled during replication initiation. Supercoiling requires the initiation factor Cdc45, as well as the single-stranded DNA-binding protein RPA, and therefore likely represents origin unwinding. When unwinding is prevented, Cdc45 binds to chromatin whereas DNA polymerase α does not, indicating that Cdc45, RPA, and DNA polymerase α bind chromatin sequentially at the G1/S transition. Whereas the extent of origin unwinding is normally limited, it increases dramatically when DNA polymerase α is inhibited, indicating that the helicase that unwinds DNA during initiation can become uncoupled from the replication fork. We discuss the implications of these results for the location of replication start sites relative to the prereplication complex.

Introduction

To insure the stable transmission of genetic information from one cell generation to the next, it is critical that origins of replication initiate DNA synthesis only once during S phase. In the last decade, a model has emerged that explains the coupling of initiation to the cell cycle (reviewed in Diffley, 1996; Leatherwood, 1998). During the G1 phase of the cell cycle, the replication initiation factors, ORC (origin recognition complex), Cdc6, and MCM (minichromosome maintenance), bind sequentially to DNA to form a prereplication complex (pre-RC). At the G1/S transition, S phase–specific cyclin-dependent protein kinase (Cdk) and Cdc7/Dbf4 kinase transform the pre-RC into an active replication fork. During this transformation, the pre-RC is disassembled with the loss of Cdc6 and MCM. Critically, MCM binding to chromatin is blocked by S and M phase–specific Cdks, preventing a new round of initiations until after Cdk activity is destroyed in mitosis.

The mechanism by which the pre-RC is transformed into an active replication fork is an area of intense interest. In addition to Cdk and Cdc7, the transformation requires the initiation factor, Cdc45, which associates with chromatin at the G1/S transition (Aparicio et al., 1997; Mimura and Takisawa, 1998; Zou and Stillman, 1998). Cdc45 binding is stimulated by Cdk (Mimura and Takisawa, 1998; Zou and Stillman, 1998), and Cdc45 is required for chromatin association of DNA polymerase α (Mimura and Takisawa, 1998; Aparicio et al., 1999). In addition, there is evidence that chromatin association of RPA, a eukaryotic single-stranded DNA-binding protein, requires Cdk and Cdc7/Dbf4, and that RPA binding precedes loading of DNA polymerase α (Tanaka and Nasmyth, 1998; see also Adachi and Laemmli, 1994). Despite these relationships, the precise order in which Cdc45, RPA, and DNA polymerase α associate with chromatin at the G1/S transition has not been clearly established.

Little is known of the specific biochemical roles that the above proteins play during initiation. Thus, while the substrate of Cdc7/Dbf4 appears to be MCM (reviewed in Leatherwood, 1998), the substrates of Cdk that must be phosphorylated to activate DNA replication are unknown. In addition, the mechanisms of origin opening and polymerase recruitment remain obscure. In comparatively simple systems such as bacteria (Kornberg and Baker, 1992) and the mammalian DNA tumor virus SV40 (Borowiec et al., 1990), the origin is opened in two discrete steps. First, an origin-bound initiator protein denatures a limited region of DNA to generate an "open complex." Second, a DNA helicase recognizes the open complex and further unwinds the DNA to generate a template recognized by DNA polymerase (Fang et al., 1999). By analogy, one might predict that ORC, the putative eukaryotic initiator, denatures the DNA to form the open complex. However, preparations of ORC that bind DNA and hydrolyze ATP show no melting activity (Klemm et al., 1997), and the proteins that initially open the origin remain unknown. Finally, the nature of the eukaryotic DNA helicases remains elusive. Although recent evidence indicates that MCM has helicase activity (You et al., 1999), it is not known what function this activity performs during initiation. For example, it is not certain whether helicase activity is required for polymerase recruitment in eukaryotes, as origin opening could be sufficient for this function.

Xenopus egg extracts represent a powerful biochemical system to study DNA replication (Blow and Laskey, 1986; Newport, 1987). These extracts assemble added sperm chromatin into nuclei that undergo a complete round of semiconservative DNA replication. In contrast to yeast, where replication initiates at defined origin sequences (reviewed in Diffley, 1996), replication in *Xenopus* egg extracts initiates at apparently random DNA sequences (Hyrien and Mechali, 1992; Mahbubani et al., 1992). Importantly, replication in egg extracts requires the initiation factors ORC, Cdc6, MCM, Cdk2, Cdc7, and Cdc45 (reviewed in Dutta and Bell 1997; Mimura and Takisawa, 1998; Tibor Roberts et al., 1999), and initiation in *Xenopus* embryos is similarly sequence independent (Hyrien and Mechali, 1993). These observations argue that the mechanism of initiation in *Xenopus* egg extracts is physiological, and for convenience, we refer to the sites where replication initiates in this system as "origins." Recently, a variation of the egg extract system has been developed in which nuclear assembly is not required for DNA replication (Walter et al., 1998). These

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"nucleus-free" extracts support extremely efficient replication of circular plasmids and thus represent a simple model system for initiation of cellular DNA replication.

To identify potential changes in the structure of origin DNA during initiation, we have examined the topology of a plasmid undergoing replication in the nucleus-free system. A marked increase in negative supercoiling was detected as plasmids underwent initiation. Supercoiling was resolved into two steps. The first step required ORC, Cdc6, MCM, Cdk2, Cdc7, and Cdc45, but not RPA, and involved a limited amount of supercoiling. In the second step, which required RPA but not Cdk2, more extensive supercoiling occurred. The RPA-dependent supercoiling likely reflects unwinding of the origin by the action of a DNA helicase. Strikingly, when DNA polymerase was inhibited with aphidicolin, massive RPA-dependent unwinding occurred, indicating that the helicase can become uncoupled from the replication fork. When unwinding was inhibited by removal of RPA or through addition of a topoisomerase inhibitor, chromatin association of DNA polymerase α was blocked while binding of Cdc45 was unaffected. These results indicate that Cdc45 binds before, RPA concurrent with, and DNA polymerase α after helicase-mediated origin unwinding.

Results

Topological Changes of a Plasmid Undergoing Replication in the Nucleus-Free System

To initiate DNA replication in the nucleus-free system, DNA is incubated with membrane-free egg cytosol to form chromatin-bound prereplication complexes containing ORC, Cdc6, and MCM (Coleman et al., 1996; Hua et al., 1997; J. W. and J. N., unpublished results). In the second step, these pre-RCs are induced to initiate DNA synthesis through the addition of a highly concentrated nucleoplasmic extract (NPE). A 3 kb plasmid such as pBluescript (pBS) replicates rapidly and with 100% efficiency in the nucleus-free system, and replication is restricted to a single round (Walter et al., 1998). Although replication initiates from random sequences on pBS (L. Sun and J. N., unpublished results), it is ORC and Cdk2 dependent.

It was shown previously that plasmids added to *Xenopus* egg cytosol are rapidly relaxed by topoisomerase I (Figure 1A; Laskey et al., 1977; Almouzni and Mechali, 1988) and then assembled into nucleosomes (Laskey et al., 1977). Because nucleosomes wrap DNA in a negative turn, nucleosome assembly leads to compensatory positive supercoiling, which is relaxed by topoisomerase I. We refer to this nucleosomal plasmid as the "ground state." After protein extraction, ground state DNA migrated at the position of supercoiled DNA near the 2 kb marker (data not shown), as expected because nucleosomes "store" negative supercoils. Figure 1B, lane 2 shows the mobility of pBS in the ground state in the presence of chloroquine. Chloroquine intercalates into DNA and locally unwinds the double helix. In a covalently closed circle, this unwinding causes compensatory positive supercoiling. Accordingly, the mobility of the negatively supercoiled ground state was decreased by chloroquine, and a distribution of topoisomers could be distinguished, indicating that the extent of nucleosome

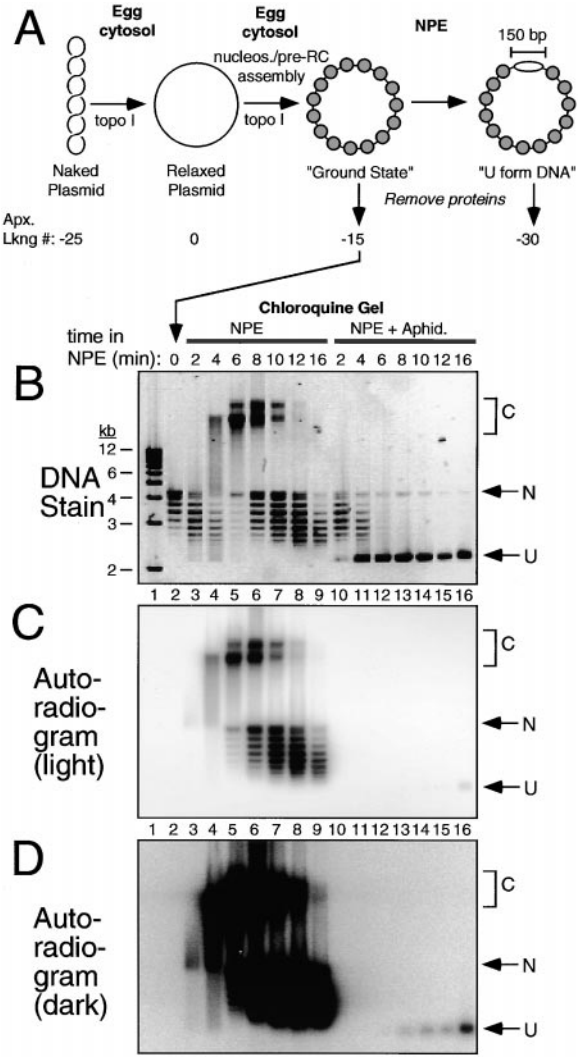

Figure 1. Topology of pBluescript Undergoing Replication in NPE

(A) Cartoon depicting the topological changes that circular plasmids undergo during incubation in egg cytosol and NPE. (B) Topological changes of replicating pBS. pBS was incubated with egg cytosol at a concentration of 40 ng/μl for 30 min, and then mixed with 2 vol of NPE supplemented with [α-^{32}P]dATP and containing (lanes 10–16) or lacking (lanes 2–9) 50 μg/ml aphidicolin. Immediately before addition of NPE, and at different times afterward, samples were removed, and the DNA was isolated, separated on a 1% agarose gel containing 1.8 μM chloroquine alongside 25 ng of a 1 kb ladder, and stained with Sybergold. Short (C) and long (D) autoradiographic exposure of the gel in (B). U, "U form DNA." N, nicked DNA.

assembly was not the same on all plasmids in the reaction (Figure 1B, lane 2).

Two minutes after the addition of NPE, there appeared a small quantity of a rapidly migrating form of the plasmid (Figure 1B, lane 3), which disappeared again by 6 min (Figure 1B, lane 5). We refer to this species as U form DNA (see below). Soon after the appearance of U form DNA, several discrete slow mobility forms of the plasmid became apparent (Figure 1B, lanes 5 and 6; form "C"). Form C likely represents daughter molecules that have partially or completely replicated but that have not yet been decatenated by topoisomerase II. Finally,

after 12–16 min, all of the plasmid returned to a topological state that was very similar to the ground state (Figure 1B; compare lanes 9 and 2). In the experiment shown in Figure 1B, the incorporation of [α-^{32}P]dATP was also measured. The autoradiogram (Figure 1C) shows that DNA synthesis is very active by the time form C appears, and quantitation of the data shows that incorporation of radioactivity ceased after 8 min (not shown). Thus, during replication of pBS in the nucleus-free system, there arise, in an ordered sequence, at least two discrete forms of the plasmid, which we call U and C, respectively.

U Form DNA Is a Bona Fide Initiation Intermediate

A key question was whether the generation of U form DNA required DNA synthesis. Replication was carried out in the presence of aphidicolin, an inhibitor of DNA polymerases α and δ. Strikingly, in the presence of aphidicolin, all of the plasmid was rapidly converted to U form DNA (Figure 1B, lanes 10–16) even though aphidicolin reduced the rate of replication ~2600-fold (Figure 1C). Importantly, the rate at which U form DNA was generated was the same with and without aphidicolin (Figure 1B, compare lanes 2–5 with lanes 10–12). Therefore, DNA synthesis is not required to generate U form DNA. We also looked at accumulation of U form DNA in the presence of 10 μg/ml actinomycin D, which inhibits DNA polymerase α–dependent replication in egg extracts by blocking the priming activity associated with DNA polymerase α (Mechali and Harland, 1982). Like aphidicolin, actinomycin D reduced DNA replication several thousand fold but had no effect on the formation of U form DNA (data not shown), indicating that generation of U form DNA does not require primer synthesis. Importantly, although accumulation of U form DNA does not require DNA or RNA synthesis, U form DNA became labeled with a small amount of radioactivity in the presence of [α-^{32}P]dATP. This is most clearly seen on a long exposure of the autoradiogram in the presence of aphidicolin (Figure 1D, lanes 10–16), and it is consistent with reports that aphidicolin does not completely block the activity of DNA polymerases (Nethanal and Kaufmann, 1990). Importantly, labeling of U form DNA was also visible in the absence of aphidicolin (Figure 1D, lanes 3 and 4). Together, these results indicate that U form DNA is a presynthetic intermediate and that DNA synthesis commences on this species.

To demonstrate rigorously that U form DNA is a bona fide replication intermediate, we performed a pulse–chase experiment. Replication was initiated using NPE that contained [α-^{32}P]dATP and AraCTP, a dCTP analog that slows the rate of DNA synthesis by DNA polymerases α and δ (Cozzarelli, 1977; Walter and Newport, 1997). As with aphidicolin, the plasmid rapidly accumulated in the U form (Figure 2A, lanes 1–5) and became labeled with radioactivity (Figure 2B, lanes 1–5). After 25 min, the reaction was supplemented with excess dCTP to reverse the inhibitory effect of the AraCTP, and with excess dATP to dilute the [α-^{32}P]dATP. As a result, the U form species rapidly disappeared, the plasmid's electrophoretic mobility decreased as it was replicated, and it ended up in the position of fully replicated product (Figure 2A, lanes 6–12). The ^{32}P incorporated during the

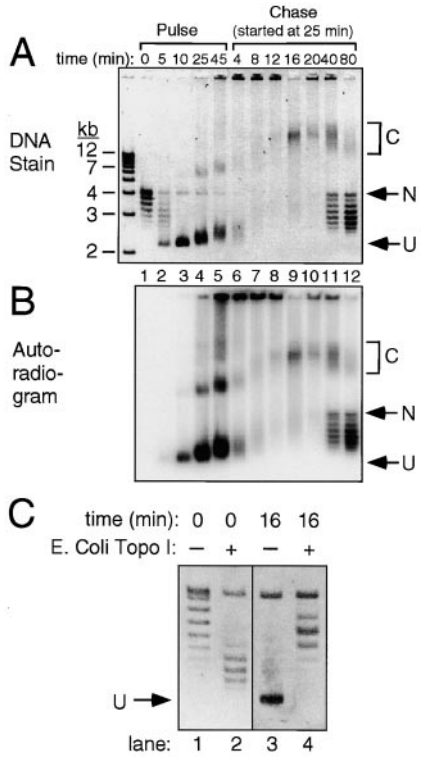

Figure 2. U Form DNA Is a Negatively Supercoiled Initiation Intermediate

(A) Pulse–chase experiment. pBS was preincubated in egg cytosol and then mixed with NPE containing 600 μM AraC and [α-^{32}P]dATP for the "pulse." Twenty-five minutes after the addition of NPE, the "chase" was initiated by adding dCTP and dATP to final concentrations of 2 and 5 mM, respectively. Samples were withdrawn at different times during the pulse (lanes 1–5) and the chase (lanes 6–12) and analyzed on a chloroquine gel as in Figure 1.

(B) Autoradiography of the gel shown in (A).

(C) U DNA and ground state DNA are negatively supercoiled. pBS was incubated in egg cytosol for 30 min and then supplemented with NPE containing 50 μg/ml aphidicolin. Samples were removed immediately before and 16 min after NPE addition. DNA was isolated, and half the sample was incubated with *E. coli* topoisomerase I (GIBCO). After reisolating the DNA, it was analyzed on a chloroquine agarose gel.

pulse experienced the same fate (Figure 2B, lanes 6–12). We conclude that U form DNA is a bona fide replication intermediate, and that the nascent chains synthesized on U form DNA are extended into fully replicated daughter molecules. These observations argue that the small amount of U form DNA seen in the absence of elongation inhibitors (Figure 1B, lanes 3 and 4) represents a steady-state level. Thus, it is continually generated by initiation and then removed as DNA synthesis commences (presumably because nascent strands reduce compaction of the U form DNA, retarding its mobility). Figures 1B and 1C also allow an estimate of the time it takes any individual plasmid to replicate: initiation (as seen by the appearance of U form DNA) is first detected 2 min after NPE addition (Figure 1B, lane 3), and the first fully replicated species found back in the ground state can be detected by autoradiography after 6 min (Figures 1C and 1D, lane 5). Thus, the maximum time needed to

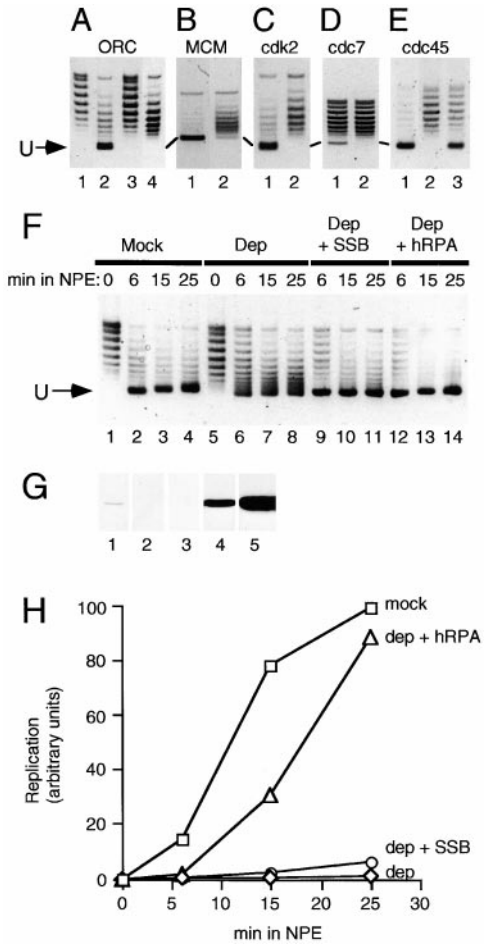

Figure 3. Factor Requirements for DNA Unwinding

(A) pBS was incubated with egg cytosol depleted of ORC (lanes 3 and 4) or mock depleted (lanes 1 and 2). Samples were withdrawn before (lanes 1 and 3) or 5 min after the addition of undepleted NPE containing aphidicolin (lanes 2 and 4). The same result was obtained with ORC-depleted NPE (data not shown).

(B) pBS was incubated with egg cytosol that had been depleted with anti-MCM7 antiserum (lane 2) or mock depleted with preimmune serum (lane 1) and then supplemented with undepleted NPE containing aphidicolin for 15 min.

(C) pBS was preincubated with undepleted egg cytosol and then mixed with NPE containing aphidicolin and 2 μM His-tagged $p21^{Cip}$ (Guadagno and Newport, 1996; lane 2) or buffer (lane 1), and unwinding measured after 4 min.

(D) pBS was incubated sequentially with egg cytosol and NPE (containing aphidicolin) that were both either depleted with Cdc7 antiserum (lane 2) or mock depleted with preimmune serum (lane 1). Samples were removed 15 min after NPE addition.

(E) pBS was incubated sequentially with egg cytosol and NPE (containing aphidicolin) that were both either depleted with anti-Cdc45 antibodies (lanes 2 and 3) or mock depleted (lane 1). The Cdc45-depleted NPE used in lane 3 was supplemented with purified His-tagged Cdc45 protein (Mimura and Takisawa, 1998) to a final concentration of 50 ng/μl. Samples were removed 15 min after NPE addition.

(F) pBS was incubated sequentially with egg cytosol and NPE that were both either mock depleted with preimmune serum (lanes 1–4) or depleted with RPA anti-serum (lanes 5–14). The NPE was supplemented with buffer (lanes 1–8), *E. coli* SSB (lanes 9–11; final concentration 450 ng/μl; Pharmacia), or recombinant human RPA (lanes 12–14; final concentration 380 ng/μl; Henricksen et al., 1994).

(G) Western blotting of RPA-depleted extracts. 0.01 μl of undepleted egg cytosol (lane 1), and 1 μl each of RPA-depleted egg cytosol (lane 2), RPA-depleted NPE (lane 3), mock-depleted egg cytosol (lane 4), or mock-depleted NPE (lane 5) were analyzed by Western blotting using antibodies against RPA. The p70 subunit of RPA is shown.

(H) DNA replication in RPA-depleted extracts. The experiment in (F) was performed in the absence of aphidicolin and the amount of DNA replication measured.

fully replicate the plasmid is 4 min, which is in good agreement with the 3 min expected to replicate a 3 kb plasmid via two forks moving in opposite directions at 500 bp/min (Mahbubani et al., 1992).

U Form DNA Is More Negatively Supercoiled Than the Ground State

We used *E. coli* topoisomerase I as a tool to probe the structure of U form DNA. This enzyme relaxes negatively but not positively supercoiled DNA (Wang, 1985). *E. coli* topoisomerase I converted both the U form DNA and the ground state DNA to a relaxed form (Figure 2C), demonstrating that both species are negatively supercoiled. Importantly, U form DNA migrates much more rapidly than ground state DNA, demonstrating that it contains more negative supercoils than the ground state. Below, we present evidence that the increase in negative supercoils reflects origin unwinding.

Factor Requirements for the Generation of U Form DNA

Initiation of DNA replication in eukaryotes requires ORC, Cdc6, MCM, Cdk, Cdc45, and Cdc7/Dbf4. It was important to determine which of these factors are required for generation of U form DNA. In the absence of ORC, no U form DNA was generated (Figure 3A, lane 4). However, we note that some supercoiling of the ground state did occur upon addition of NPE (Figure 3A, compare lanes 3 and 4), but the ORC independence of this phenomenon indicates that it is not related to initiation, and we speculate that it may reflect further nucleosome assembly in NPE. Next, we examined the requirement for MCM. To block replication, it was sufficient to remove MCM from egg cytosol because NPE contains inhibitors that prevent de novo binding of MCM to chromatin (Walter et al., 1998; J. W. and J. N., unpublished results), and under these circumstances there was also no unwinding (Figure 3B). Next, we examined the requirement for the kinases Cdk2 and Cdc7 in unwinding and found that both are required (Figures 3C and 3D). Finally, removal of Cdc45 from extracts inhibited unwinding (Figure 3E, lane 2), and the inhibition was reversed with purified recombinant Cdc45 (Figure 3E, lane 3). In all of these experiments, we found that when unwinding was inhibited, replication was also blocked (data not shown). Although it was not possible in all cases to add back purified protein to rescue the effect of depletion, the fact that chromatin binding of Cdc45 appears to require MCM, Cdk2, and Cdc7/Dbf4 (Owens et al., 1997; Mimura and Takisawa, 1998; Zou and Stillman, 1998), and by inference, ORC and Cdc6 (Coleman et al., 1996), strongly supports our conclusion that each of these initiation factors executes at least one of its functions upstream of the unwinding step.

We also examined the role of the eukaryotic single-stranded DNA-binding protein, RPA, in the generation of U form DNA. When the amount of RPA in egg cytosol and NPE was reduced to less than 1% the level normally found in egg cytosol (Figure 3G), there was no DNA replication (Figure 3H, diamonds), and the amount of U form DNA generated was greatly reduced (Figure 3F, lanes 6–8). Both replication and generation of U form DNA were rescued by addition of recombinant human RPA (Figure 3H, triangles, and Figure 3F, lanes 12–14). Importantly, U form DNA could also be generated when RPA was substituted with *E. coli* single-stranded DNA-binding protein (SSB; Figure 3F, lanes 9–11), even though SSB did not support DNA replication (Figure 3H, circles). The data support the idea that U form DNA represents an unwound form of the origin that is stabilized by RPA or SSB. This unwinding causes compensatory positive supercoiling, which is relaxed by topoisomerase I. Thus, the plasmid gains one negative supercoil for every 10 bp of unwound DNA, resulting in U form DNA. Based on the precedent from the SV40 system, we suggest that RPA-mediated unwinding is carried out by a DNA helicase.

Interestingly, although efficient generation of U form DNA required RPA, there was a significant increase in supercoiling that occurred in an RPA-independent fashion (Figure 3F, lanes 5–8). We do not believe that this shift reflects residual RPA. First, RPA has been reduced to such low levels that there is no DNA replication. Second, at this level of depletion, there is no RPA binding to chromatin (Figure 5D). Third, the shift still occurs when RPA-depleted extracts are challenged with an excess of single-stranded M13 DNA to compete any residual single-stranded DNA binding activities (data not shown). In the Discussion, we explore possible mechanisms for RPA-independent supercoiling.

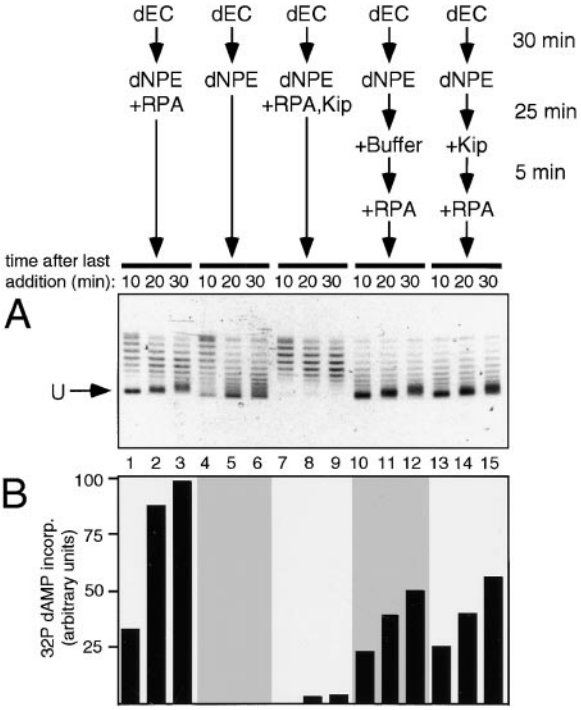

Figure 4. Cdk2 Is Not Required for RPA-Dependent DNA Unwinding

(A) pBS was incubated with 3 μl RPA-depleted egg cytosol for 30 min and then supplemented with 9 μl RPA-depleted NPE containing aphidicolin. After 25 min, either buffer (10–12) or $p27^{kip}$ was added to a final concentration of 1.7 μM (13–15). After 5 min, 2 μl of undepleted NPE was added to supply RPA, and unwinding was measured. In controls, unwinding was measured directly after addition of RPA-depleted NPE (containing aphidicolin) that was not supplemented with anything (4–6), supplemented with 2 μl undepleted NPE (1–3), or 2 μl undepleted NPE and $p27^{Kip}$ (7–9).

(B) The same reactions as in (A) were carried out in the absence of aphidicolin, and DNA replication was measured.

Cdk2 Activity Is Not Required for RPA-Dependent Unwinding

Our data indicate that in the absence of ORC, Cdc6, MCM, Cdk2, Cdc7, or Cdc45, there is no DNA unwinding. The question arises as to whether these factors function directly in RPA-mediated unwinding (as a helicase or helicase cofactor), or at an upstream step such as the RPA-independent supercoiling event. We have addressed this question for Cdk2. Plasmid was incubated in egg cytosol and NPE lacking RPA to advance past the RPA-independent step (Figure 4A, lanes 4–6). Next, the Cdk2 inhibitor $p27^{Kip}$ was added. After 5 min, undepleted NPE was added to supply RPA, and unwinding was measured in the presence of aphidicolin. We found that $p27^{Kip}$ did not hinder unwinding relative to a negative control lacking $p27^{Kip}$ (Figure 4A, compare lanes 13–15 with 10–12), and the extent of unwinding was at least as much as in a control that contained RPA from the start (Figure 4A, lanes 1–3). In contrast, when $p27^{Kip}$ was added with the NPE at the start, it blocked unwinding before the RPA-independent step (Figure 4A, lanes 7–9). Together, these results indicate that once the RPA-independent step has occurred, there is no further requirement for Cdk2 activity during unwinding. The same experiment as in Figure 4A was carried out in the absence of aphidicolin (Figure 4B). Again, $p27^{Kip}$ had no inhibitory effect when added after the RPA-independent step (compare Figure 4B, lanes 13–15 and 10–12), indicating that once this step has occurred, Cdk2 activity is also not required for DNA replication.

DNA Unwinding Is Necessary for Chromatin Binding of DNA Polymerase α but Not Cdc45

We next investigated how unwinding relates to other events that occur at the G1/S transition. In yeast, and in reconstituted *Xenopus* nuclei, the G1/S transition is marked by Cdk-dependent stable association of Cdc45, RPA, and DNA polymerase α with chromatin (Fang and Newport, 1993; Adachi and Laemmli, 1994; Aparicio et al., 1997, 1999; Mimura and Takisawa, 1998; Tanaka and Nasmyth, 1998; Zou and Stillman, 1998). Similarly, in the nucleus-free system, addition of NPE to pre-RCs formed in egg cytosol led to the rapid chromatin association of Cdc45, RPA, and DNA polymerase α (Figure 5A, lanes 2 and 3). Binding of these factors required the activity of Cdk2 since it is blocked by addition of $p27^{Kip}$ (Figure 5A, lanes 4 and 5). The small amount of Cdc45 bound in the presence of $p27^{Kip}$ may be analogous to the weak Cdk-independent "bound state" of Cdc45 proposed in yeast (Aparicio et al., 1997, 1999). Interestingly, Cdc45

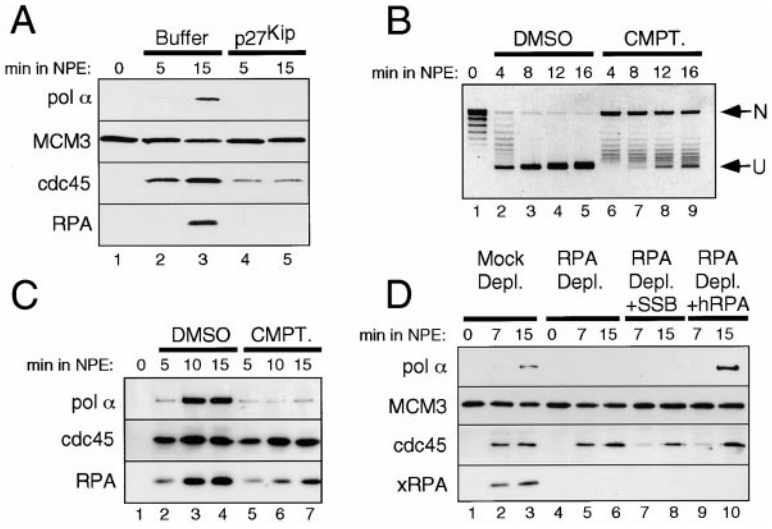

Figure 5. RPA-Dependent Unwinding Is Required for Loading of DNA Polymerase α but Not Cdc45

(A) Binding of Cdc45, RPA, and DNA polymerase α to chromatin at the onset of S phase in nucleus-free egg extracts. Sperm chromatin was incubated with egg cytosol for 30 min and then supplemented with NPE, containing (lanes 4–5) or lacking (lanes 2–3) $p27^{kip}$. Samples were withdrawn, and chromatin was isolated and analyzed by Western blotting using antibodies against the 180 kDa subunit of DNA polymerase α (Dornreiter et al., 1993), MCM3 (Hua et al., 1997), Cdc45 (Mimura and Takisawa, 1998), and the 34 kDa subunit of *Xenopus* RPA (Fang and Newport, 1993).

(B) Camptothecin inhibits unwinding. pBS was preincubated in egg cytosol and then mixed with NPE + aphidicolin that also contained DMSO (lanes 2–5) or camptothecin at 0.5 mM (lanes 6–9).

(C) Camptothecin inhibits DNA polymerase α but not Cdc45 binding to chromatin. Same as (B) except that sperm chromatin was used instead of plasmid, and chromatin-bound proteins were isolated and analyzed by Western blotting as in (A).

(D) RPA is required for chromatin binding of DNA polymerase α, but not Cdc45. Sperm chromatin was incubated sequentially with egg cytosol and NPE that were both either mock depleted with preimmune serum (lanes 1–3) or RPA depleted using RPA antiserum (lanes 4–10). The NPE used was supplemented with buffer (lanes 1–6), *E. coli* SSB (lanes 7 and 8; final concentration 470 ng/μl), or recombinant human RPA (lanes 9 and 10; final concentration 370 ng/μl).

appears to load onto DNA before RPA and DNA polymerase α, suggesting that Cdc45 and DNA polymerase α do not bind to chromatin as a complex as previously proposed (Mimura and Takisawa, 1998).

To examine the role of DNA unwinding in the chromatin association of Cdc45, RPA, and DNA polymerase α, we sought to specifically inhibit the unwinding reaction. Topoisomerase activity is expected to be required indirectly for unwinding because the positive superhelical stress generated during unwinding must be relieved (Dodson et al., 1987). Therefore, we used camptothecin, an inhibitor of topoisomerase I that was previously shown to inhibit topoisomerase activity in *Xenopus* egg extracts (Almouzni and Mechali, 1988). Camptothecin dramatically reduced the amount of U form DNA generated in the presence of aphidicolin (Figure 5B, lanes 6–9). Camptothecin converted a substantial portion of the plasmid to the nicked circular form, consistent with previous observations that this drug traps topoisomerase I in a complex with DNA in which one strand is cleaved (Pommier et al., 1998). Importantly, camptothecin also strongly reduced the association of RPA and DNA polymerase α with sperm chromatin (Figure 5C, lanes 5–7). In contrast, the binding of Cdc45 was unaffected (Figure 5C, lanes 5–7). It is important to note that the inhibitory effect of camptothecin on chromatin binding and replication was transient, lasting ~20 min (data not shown). We suggest that its function was taken over by topoisomerase II, which is also present in egg extracts (Hirano and Mitchison, 1993). To obtain independent evidence for the function of unwinding during initiation, we also examined chromatin binding of Cdc45 and DNA polymerase α in RPA-depleted extracts because unwinding does not occur in these extracts (Figure 3F). DNA polymerase α did not bind to chromatin in the absence of RPA (Figure 5D, lanes 5 and 6), and the binding could be restored with the addition of recombinant human RPA (Figure 5D, lanes 9 and 10). Binding of Cdc45 was normal in the absence of RPA (Figure 5D, lanes 5 and 6). Thus, RPA depletion and camptothecin inhibition experiments both yield the same conclusion: DNA unwinding is critical for loading of the initiating DNA polymerase whereas it is not required for binding of Cdc45. Interestingly, when the RPA-depleted extract was supplemented with *E. coli* SSB, there was no rescue of DNA polymerase α binding (Figure 5D, lanes 7 and 8), even though SSB supports DNA unwinding (Figure 3F). Together, these results indicate that Cdc45, RPA, and DNA polymerase α bind to origins of replication one after another at the G1/S transition.

A Limited Amount of DNA Is Unwound during Initiation

We next asked how much DNA is unwound during initiation. To this end, we compared the electrophoretic mobility of U form DNA with two standards. Plasmid was preincubated in egg cytosol, and NPE lacking replication inhibitors was added to generate U form DNA. At different times after addition of NPE, the plasmid was isolated and subjected to electrophoresis in the presence of increasing concentrations of chloroquine (Figures 6A–6E, lanes 1–3). On the same gel, we analyzed a plasmid that was prevented from initiating by $p27^{Kip}$ (Figures 6A–6E, lane 4) and a standard (pBS that was never exposed to extract; Figures 6A–6E, lane 6). As shown by an enlargement of the gel in Figure 6F, the most negatively supercoiled topoisomer in the standard (closed arrowhead) contains nine more negative supercoils than the average topoisomer in the uninitiated plasmid preparation (open arrowhead; see figure legend). Importantly, at 1.8 μM chloroquine, the standard already begins to migrate up the gel (Figure 6A, lane 6), whereas U form

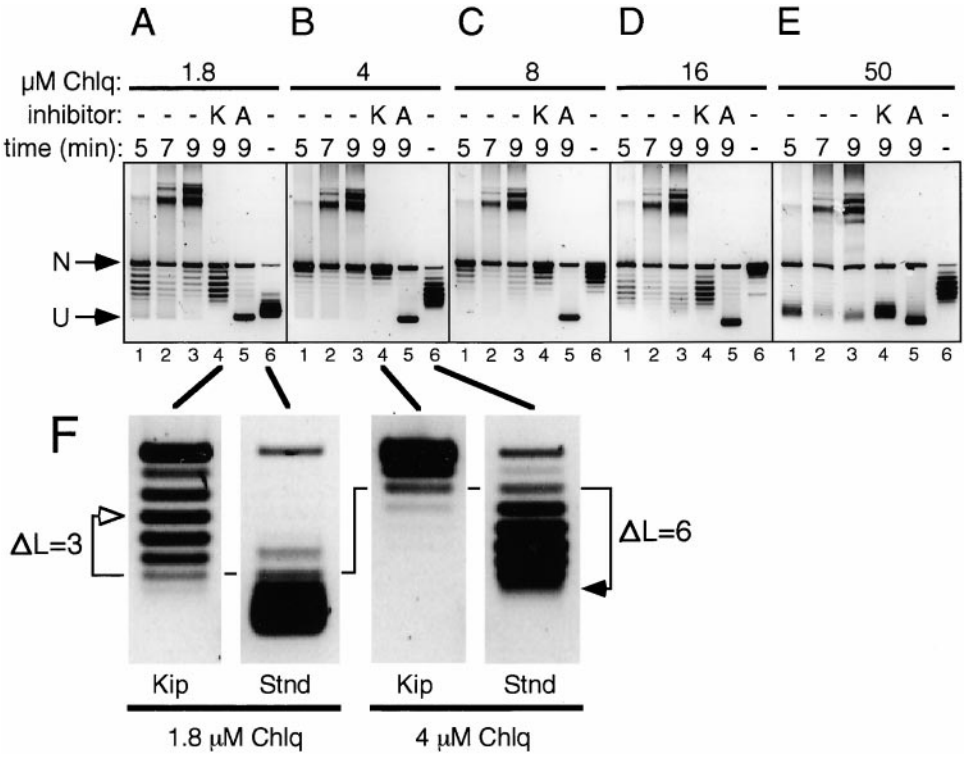

Figure 6. Extent of DNA Unwinding in the Presence and Absence of Aphidicolin

(A–E) Electrophoretic mobility of U form DNA generated in the presence and absence of aphidicolin at different chloroquine concentrations. pBS was preincubated with egg cytosol for 30 min and then supplemented with NPE containing no additives (lanes 1–3), 2 μM $p27^{Kip}$ (lane 4), or 50 μg/ml aphidicolin (lane 5). DNA was isolated at different times after the addition of NPE and analyzed on agarose gels containing increasing concentrations of chloroquine alongside 40 ng of supercoiled pBS that was never exposed to extract (lane 6).

(F) Enlargement of selected lanes from (A) and (B). The double-headed arrow goes from the average species on the uninitiated plasmid at 1.8 μM chloroquine (open arrowhead) to the most supercoiled species in the standard at 4 μM chloroquine (closed arrowhead), and it spans 9 supercoils ($\Delta L = 3 + 6 = 9$).

DNA still migrates at the position of fully supercoiled DNA (Figure 6A, lane 1). Therefore, U form DNA contains at least nine more negative supercoils than the uninitiated plasmid. At 4 μM chloroquine, approximately half the U form DNA has started to migrate up the gel (Figure 6B, lane 1), and in 8 μM chloroquine, no DNA is left in the position of U form (Figure 6C, lane 1). Therefore, it takes about twice as much chloroquine to move the bulk of the U form DNA compared to the standard. In this concentration range, a doubling of the chloroquine concentration changes the mobility of the plasmid by roughly 5 linking numbers (compare the standard at 1.8 and 4 μM chloroquine in Figure 6F). We conclude that the bulk of the U form DNA contains ~15 more negative supercoils than the uninitiated plasmid. Although these data are not highly quantitative, they indicate that the amount of DNA unwound during initiation is limited (see Discussion).

Uncoupling of the Helicase from the Replication Fork

We were also interested in the minimum amount of DNA that must be unwound during initiation to load DNA polymerase α onto chromatin. We therefore measured the extent of unwinding in the presence of aphidicolin because we expected that aphidicolin might arrest the replication fork as soon as the polymerase is loaded. Strikingly, the mobility of U form DNA generated in the presence of aphidicolin did not change, even when it was exposed to chloroquine concentrations up to 50 μM (Figures 6A–6E, lane 5). This demonstrates that when the polymerase is inhibited, the extent of DNA unwinding is dramatically increased. Based on a comparison of our results with unwinding in bacterial and SV40 DNA replication (see Experimental Procedures), we estimate that U form DNA contains several kilobase pairs of single-stranded DNA. The extensive nature of this unwinding strongly suggests that RPA-dependent unwinding events involve the action of a DNA helicase, which can become uncoupled from the polymerase.

Discussion

A Model for Origin Unwinding

We have identified several distinct topological states of a circular plasmid (pBS) undergoing replication initiation in *Xenopus* egg extracts (Figure 7). When pBS is incubated in egg cytosol, pre-RCs are assembled, and the plasmid is packaged into nucleosomal DNA ("ground state," Figure 7). Upon addition of NPE, initiation-dependent negative supercoiling occurs in two steps. First, limited supercoiling that requires ORC, Cdc6, MCM,

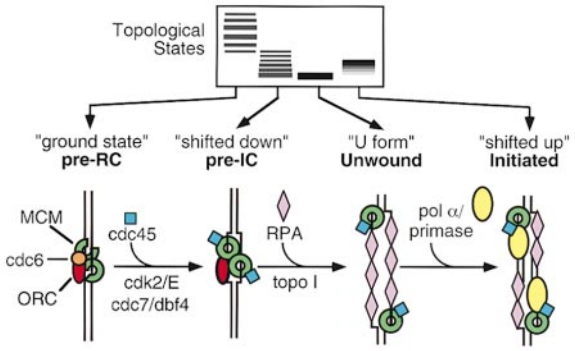

Figure 7. Model for Initiation of Eukaryotic DNA Replication

Cdk2, Cdc7, and Cdc45 but not RPA occurs ("shifted down" species). This event coincides with the binding of Cdc45 to chromatin to form pre-ICs. Subsequently, more extensive, RPA-dependent supercoiling occurs (U form DNA). Finally, initiation of DNA synthesis gives rise to slower mobility replication intermediates ("shifted up" species).

What is the nature of the supercoiled intermediates? There are at least two interpretations of the RPA-independent supercoiling event that accompanies pre-IC formation. One is that DNA is wrapped in a negative turn around Cdc45 or other initiation factors at the G1/S transition. The second possibility is that RPA-independent supercoiling reflects helicase-independent opening of the origin analogous to open complex formation at *oriC* (Figure 7; Baker and Kornberg, 1992). Further work will be necessary to distinguish between these possibilities.

Because it requires a single-stranded DNA-binding protein, the RPA-*dependent* supercoiling step almost certainly reflects unwinding of the origin during initiation. The extensive unwinding that occurs in the presence of aphidicolin argues that unwinding reflects the action of a DNA helicase. We find that in the absence of MCM, a putative eukaryotic replicative DNA helicase (Aparicio et al., 1997; You et al., 1999), there is no unwinding. However, the block occurs before the RPA-*independent* supercoiling step. Therefore, further work will be needed to determine whether MCM is *directly* required for RPA-mediated unwinding. In the case of Cdk2, we have shown that it is not directly required for unwinding. Furthermore, after binding of MCM to the chromatin in egg cytosol, ORC and Cdc6 become dispensable for replication (Hua and Newport, 1998; Rowles et al., 1999). Because unwinding only occurs after addition of NPE, these data imply that ORC and Cdc6 are also not directly required for unwinding. Finally, like T antigen–mediated unwinding (Dean et al., 1987; Wold et al., 1987), unwinding in our system is supported by *E. coli* SSB, indicating that there are no specific protein–protein interactions between RPA and the cellular DNA helicase. Our results support previous suggestions that RPA bound to chromosomes at the G1/S transition in vitro (Adachi and Laemmli, 1994) and in vivo (Tanaka and Nasmyth, 1998; Dimitrova et al., 1999) reflects unwinding of replication origins.

Mechanism of Loading DNA Polymerase α

Our results indicate that RPA-mediated unwinding of the origin is critical for recruitment of DNA polymerase α. Because DNA polymerase α is tightly complexed with primase subunits in a polymerase/primase holoenzyme (Dornreiter et al., 1990), this conclusion likely also applies to the primase. The ability of *E. coli* SSB to support unwinding but not DNA polymerase α loading shows that unwinding is not sufficient for recruitment and suggests that specific protein–protein contacts between RPA and the DNA polymerase α/primase complex (Dornreiter et al., 1992) might be important. The requirement for RPA-mediated origin unwinding in loading DNA polymerase α was previously suggested by in vivo cross-linking studies performed in hydroxyurea-arrested yeast cells (Tanaka and Nasmyth, 1998).

Several lines of evidence suggest that DNA polymerase α binds specifically to the unwinding fork rather than anywhere within the unwound complex. Thus, single-stranded M13 DNA can be replicated by DNA polymerase α in egg cytosol but not in NPE (J. W. and J. N., unpublished results), indicating that there is a nucleus-specific mechanism that suppresses initiation events on any single-stranded DNA. In addition, interactions between DNA polymerase α and several proteins believed to be located at the replication fork have been reported (Aparicio et al., 1997; Mimura and Takisawa, 1998). Finally, the ability to remove ORC and Cdc6 from chromatin before initiation (Hua and Newport, 1998; Rowles et al., 1999) argues that DNA polymerase α does not bind to the pre-RC. These considerations suggest that replication will initiate at a distance from the pre-RC that is equivalent to the extent of unwinding (see below).

Cdc45, RPA, and DNA Polymerase α Bind Chromatin One after the Other at the G1/S Transition

It was shown previously that Cdc45 is required for loading of DNA polymerase α to chromatin in yeast and in conventional *Xenopus* egg extracts (Mimura and Takisawa, 1998; Aparicio et al., 1999). Mimura and Takisawa (1998) proposed that these two factors bind chromatin as a complex, whereas the work in yeast suggested that they bind the origin separately (Tanaka and Nasmyth, 1998; Zou and Stillman, 1998; Aparicio et al., 1999). Our data clearly support the latter view. We find that when unwinding is prevented by removal of RPA or by the addition of the topoisomerase I inhibitor camptothecin, binding of DNA polymerase α is blocked whereas binding of Cdc45 is unaffected. Our observation that *E. coli* SSB is able to unwind the origin in the absence of bound DNA polymerase α indicates that single-stranded DNA-binding proteins can bind the origin in the absence of polymerase. Together, these results argue that Cdc45, RPA, and DNA polymerase α bind chromatin one after another during initiation (Figure 7). The sequential chromatin binding of these three factors has also been observed in conventional *Xenopus* egg extracts (Mimura et al., 2000). Importantly, we have also shown that Cdc45 is required for unwinding. Thus, the requirement for Cdc45 in loading DNA polymerase α is adequately explained in terms of the involvement of Cdc45 in DNA unwinding.

How Much DNA Is Unwound during Initiation?

Two-dimensional gels (Hyrien and Mechali, 1992) and electron microscopy (Lucas et al., 2000) indicate that small plasmids replicating in *Xenopus* nuclear assembly extracts contain a single replication bubble, implying a single initiation event. Plasmids are expected to replicate by the same mechanism in the nucleus-free system because the licensing reaction is the same as in the nuclear assembly system. Moreover, the kinetics of replication in the absence of nuclei are exactly what is expected from replication by two forks (Figure 1B). Therefore, our estimate that a plasmid acquires about 15 negative supercoils at the G1/S transition would appear to suggest that roughly 150 base pairs of DNA are unwound during a single initiation event. However, it cannot be ruled out that initiation in egg extracts occurs from several closely spaced origins that later fuse, nor can we exclude the possibility that some of the supercoiling observed reflects conformational changes in DNA not related to origin unwinding. Therefore, 150 base pairs likely represents an *upper limit* of how much DNA is unwound during a single initiation. Nevertheless, this number is highly significant because it argues strongly against models in which many kilobase pairs of DNA are unwound before DNA synthesis commences (Benbow et al., 1992).

Uncoupling of the Helicase from the Replication Fork

In the presence of aphidicolin, we observed massive DNA unwinding. A similar phenomenon occurs when initiation is uncoupled from elongation in the SV40 and OriC systems (Baker et al., 1986; Dean et al., 1987). Thus, it appears that a universal feature of replicative DNA helicases is that they can become uncoupled from the replication fork. Extensive unwinding is not a peculiarity of using plasmids as DNA substrates because aphidicolin also markedly increased the amount of RPA that bound to sperm chromatin after addition of NPE (J. W. and J. N., data not shown). Interestingly, when G1 nuclei from hamster cells are incubated with *Xenopus* egg extract, aphidicolin induces a dramatic broadening of the initiation zone at the *DHFR* locus (Li et al., 2000). We suggest that aphidicolin induces helicase uncoupling in these cells and that the broad initiation zone results because the helicase has unwound the DNA to different extents. If this interpretation is correct, these results indicate that uncoupling can occur in the context of somatic chromatin structure.

The question arises as to how the helicase and polymerase are normally coupled, and how aphidicolin overcomes this coupling. One possibility suggested by work in *E. coli* (Kim et al., 1996) is that the helicase moves at a slower intrinsic rate than the polymerase, insuring that the helicase never moves ahead of the polymerase. In this scenario, aphidicolin would induce uncoupling simply by stalling the polymerase. Another possibility is that the helicase is physically tethered to some component of the replication fork, preventing it from "escaping." How might aphidicolin circumvent this coupling mechanism? Perhaps the helicase is normally tethered to the replication fork via a factor, such as DNA polymerase δ, that only binds to the DNA after DNA polymerase α has made a DNA primer. Further work will be required to distinguish between these possibilities.

Our demonstration that DNA unwinding is necessary for polymerase recruitment, and that the helicase can apparently unwind DNA in the absence of polymerase activity, has potential implications for the distribution of replication start sites. On the one hand, if recruitment of the polymerase is diffusion driven, there may be microheterogeneity in the distribution of replication start sites at all origins. Such microheterogeneity has been observed in *oriC*, SV40, and other noneukaryotic replication systems (Fang et al., 1999, and references therein), and it was proposed that it arises because the polymerase must capture a moving helicase target (Fang et al., 1999). On the other hand, more extensive unwinding might occur at loci where chromatin structure favors rapid helicase movement or where DNA polymerase recruitment to the DNA is slow. Interestingly, replication normally initiates at a precise point at the chromosomal *ARS1* origin in yeast (Bielinsky and Gerbi, 1999), whereas when *ARS1* is placed on an episome, initiations occur within a more diffuse region (Bielinsky and Gerbi, 1998). We speculate that the helicase may move more rapidly on the episome or that the episome lacks structures such as replication factories (Pasero et al., 1997) that might couple polymerase recruitment to origin unwinding. In mammalian cells, replication is sometimes found to initiate throughout broad regions, even in the absence of aphidicolin (DePamphilis, 1999). Although this observation may be due to a broad distribution of pre-RCs, it may also reflect movement of the helicase away from the pre-RC. In the future, it will be challenging to assess what contribution DNA unwinding makes to the distribution of replication start sites in vivo.

Experimental Procedures

Plasmid DNA Replication

Supercoiled pBluescript II KS (−) was replicated in the nucleus-free system (Walter et al., 1998) at a final concentration of 13 ng/μl. To measure replication, 0.1 μCi/μl [α-^{32}P]dATP was added. Typically, 3 μl samples were withdrawn, mixed with 97 μl stop buffer (0.5% SDS, 20 mM EDTA), supplemented with 2 μl 20 mg/ml proteinase K, and incubated for 30 min at 37°C. DNA was then extracted and ethanol precipitated.

Electrophoresis

Unless stated otherwise, 20–30 ng of plasmid isolated after replication was separated on 1.0% TBE agarose gels containing 1.8 μM chloroquine phosphate at 4.25 V/cm for 3 hr. Subsequently, the gel was stained with 1:10,000 Sybergold (Molecular Probes) in TBE for 60 min. The gel was photographed, scanned into photoshop, and inverted, and the contrast was adjusted to best match the original photograph. To measure replication, the gel was dried supported by DEAE paper and exposed to a phosphorimager plate (Molecular Dynamics).

When elongation is uncoupled from initiation using a 4 kb *oriC*-containing plasmid, there is a large extent of DNA unwinding (Baker et al., 1986). Electron microscopy revealed that $\sim$2 kb of this plasmid was unwound (200 negative supercoils). This highly unwound species did not move from the position of supercoiled DNA during gel electrophoresis in Tris-phosphate buffer (TPE) containing 500 μM chloroquine. We find that 500 μM chloroquine in TPE is equivalent to 50 μM chloroquine in TBE (data not shown), the buffer system used in the present study. Since U form DNA generated in the presence of aphidicolin did not move up the gel in 50 μM chloroquine/TBE (Figure 6), we conclude that this species is unwound

to an extent that is similar to the unwound species characterized by Baker et al. (1986).

Immunodepletion, Antibodies, and Chromatin Binding

To specifically remove proteins from egg cytosol or NPE, we typically used three consecutive immunodepletions as described previously (Walter and Newport, 1999). In the case of Cdc45, 1 μg affinity-purified anti-Cdc45 antibody (Mimura and Takisawa, 1998) was used for each round of depletion. For all other proteins, monospecific sera were used for the depletion, and preimmune sera were used for mock-depletion controls. Anti-xORC2 serum was as previously described (Walter and Newport, 1997). RPA antiserum was raised against the RPA heterotrimer purified from *Xenopus* egg cytosol as described previously (Fang and Newport, 1993), and on Western blots it specifically recognized the 70 kDa, 34 kDa, and 14 kDa subunits of RPA. Cdc7 antiserum was raised against His-tagged Cdc7 protein (Sato et al., 1997), which was expressed from a pET28a+ vector in *E. coli* and purified using Ni-affinity chromatography using standard techniques. The anti-Cdc7 antiserum specifically recognized a ~55 kDa protein in Western blots. MCM7 antiserum was raised against a bacterially expressed C-terminal fragment of MCM7 (amino acids 545–720) that was purified using Ni-affinity chromatography. The antiserum specifically recognized a protein of molecular weight ~100 kDa. In all cases, the extent of depletion was monitored by Western blotting and found to be at least 99%. Chromatin binding assays were performed as previously described (Walter and Newport, 1997, 1999).

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