De novo assembly of genuine replication forks on an immobilized circular plasmid in *Xenopus* egg extracts

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ABSTRACT

We describe an improved model of DNA replication in *Xenopus* egg extracts, in which a circular plasmid immobilized on paramagnetic beads is used as a template. DNA synthesis occurred on either circular or linear plasmids coupled to the beads, but only DNA synthesis on the circular plasmid was inhibited by geminin and a CDK inhibitor, p21. DNA synthesis on the circular plasmid occurred after a time lag, during which nuclear formation was probably occurring. Although pre-replicative complexes (pre-RCs) were formed soon after mixing plasmids with egg extracts, binding of CDC45, RPA, Pol α, δ and ε, and PCNA to the circular plasmid was delayed, but still correlated with DNA synthesis. Moreover, p21 inhibited binding of these replication fork proteins to the circular plasmid. Therefore, the circular plasmid, but not the linear plasmid, assembles bona fide replication forks in egg extracts. We conclude that this improved replication system will be useful for studying the mechanism of formation of replication forks in eukaryotic DNA replication.

INTRODUCTION

DNA replication in eukaryotes is initiated through the coordinated actions of replication initiation proteins. Studies in yeast and other species have identified many initiation proteins and revealed the outline of the mechanism through which DNA replication begins in eukaryotic cells (1–3). In brief, an origin recognition complex (ORC) consisting of the ORC1–6 subunits binds to DNA and then, in cooperation with CDC6 and CDT1, loads a mini-chromosome maintenance (MCM) 2–7 protein complex on to the DNA to form a specific protein–DNA complex, which is called the pre-replicative complex (pre-RC). MCM2–7 is thought to function as a DNA helicase during the initiation and elongation steps (4–9). Geminin is known to inhibit pre-RC formation during S, G_2 and M phases of the cell cycle and thus is considered as the major regulator of pre-RC formation during the cell cycle (2,10). However, although the basic mechanism of pre-RC formation is understood, many details remain to be defined. After formation, the pre-RC is activated in a poorly understood process that involves many factors, including cyclin-dependent kinase (CDK) and CDC7 kinase, and activation leads to replication fork formation (1,11–17).

To study the details of pre-RC formation and activation, a biochemical approach using *in vitro* analysis of the pre-RC is needed. Several studies have taken such *in vitro* approaches, mainly through analysis of the pre-RC in budding yeast (18–20). This approach takes advantage of a property unique to budding yeast among the various species characterized to date: that the ORC binds to a well-characterized, defined replication origin, the so-called autonomously replicating sequence (ARS) (21). Among these studies, the experimental system reported by Seki and Diffley (19) appears to be especially useful for analysis of the pre-RC. In this system, the pre-RC can be successfully reconstituted on an ARS sequence coupled to paramagnetic beads, and the system can then be used to address the role of ATP hydrolysis by the yeast ORC in pre-RC formation (18).

Although *in vitro* replication using yeast nuclear extracts has been reported (22,23), the exact activity of the reconstituted pre-RC in the above studies is unknown. In contrast, the *Xenopus* replication system is able to replicate various kinds of DNA efficiently, whereas initiation generally occurs randomly with respect to the DNA sequence (24–28). However, recent studies have established the conditions for initiation at a specific DNA region, using methylated DNA (29) or transcription factor-bound DNA (30), with one such study showing that MCM loaded on to DNA is distributed over a large region distant from the ORC (29). In addition to free plasmids, DNA synthesis on DNA-coupled beads has also been performed with cell extracts of *Xenopus* eggs, and used to analyze activities such as pre-RC formation, chromatin assembly and spindle assembly (31–35). Using circular plasmid-coupled magnetic beads, we recently showed that only one or a few MCM complexes may be loaded per ORC DNA-binding event (36), but DNA synthesis on the beads in the egg extracts has not been fully characterized.

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Here, we report the characterization of DNA replication on plasmid-coupled magnetic beads in *Xenopus* egg extracts. Our data show that DNA replication occurs in a pre-RC- and CDK-dependent manner when a circular plasmid is coupled to magnetic beads and then incubated in *Xenopus* egg extracts. Furthermore, we were able to detect regulated DNA binding of replication fork proteins, including three different DNA polymerases, suggesting that bona fide replication forks are formed *de novo* on the circular plasmid. Therefore, this DNA replication model based on plasmid immobilization on beads is likely to be useful for the analysis of pre-RC activation.

MATERIALS AND METHODS

Materials

Wheat germ agglutinin (WGA) was purchased from Wako (Osaka, Japan).

Plasmid

The plasmids used as templates were pG5λ6.6 (11 kb), pEXλ6.6 (12 kb), pKS-EX (5 kb) and pBluescript SK(−) (3 kb) (Stratagene). pG5λ6.6 is a pGEM-7Zf(+) (Promega)-based plasmid that contains five Gal4-binding sites, an adenovirus major late promoter and a 6.6 kb λDNA/HindIII fragment. pKS-EX is a pBluescript KS-based plasmid that contains a 2.2 kb Epstein–Barr virus ori P sequence (a gift from Dr Masaki Shirakata, Tokyo Medical and Dental University, Japan) and pEXλ6.6 was constructed by inserting a 6.6 kb λDNA/HindIII fragment into pKS-EX. The plasmids were prepared using a Genopure Plasmid kit (Roche).

Biotinylation of plasmids and binding to superparamagnetic beads

Circular plasmid DNA was biotinylated by photocoupling with Photoprobe (S–S) Biotin (Vector Laboratories), which has a cleavable disulfide bond in the linker arm. The procedure was performed according to the manufacturer's instructions. Briefly, 100 μg of plasmid was mixed with 0.5 μg of photoreactive biotin and irradiated for 10 min with a 365 nm UV lamp (8 W). The coupling mixture was mixed with an equal volume of 0.1 M Tris–HCl (pH 9.5) and extracted with 2-butanol to remove free photoreactive biotin. The biotinylated plasmid was then precipitated with ethanol.

The biotinylated plasmid (10 μg) was bound to 400 μg of streptavidin–Dynabead M-280 (Dynal Biotech), with the binding reaction carried out using a Dynabeads kilobaseBINDER kit (Dynal Biotech) according to the manufacturer's instructions. Typically, ~60 ng of circular plasmid was coupled to 10 μg of beads. To biotinylate linearized pG5λ6.6, the plasmid was linearized by digestion with SacI and then subjected to an exchange reaction with T4 DNA polymerase in the presence of 100 μM biotin-16-dUTP (Roche) and 200 μM dATPαS (GE Healthcare Bio-Sciences). In this reaction, biotin-16-dUMP was incorporated at one end of the plasmid and dAMPαS was incorporated at the other end. Incorporation of dAMPαS was used to block exonuclease activity (37). To biotinylate linearized pEXλ6.6, the plasmid was linearized by digestion with KpnI and then subjected to an exchange reaction with T4 DNA polymerase in the presence of biotin-16-dUTP and dCTP.

Antibodies and recombinant proteins

The rabbit anti-*Xenopus* ORC1 and ORC2 antibodies were raised against bacterially expressed, histidine-tagged recombinant proteins. Rabbit anti-*Xenopus* MCM3, MCM6 and CDC45 antibodies were gifts from Dr Haruhiko Takisawa (Osaka University, Japan); rabbit anti-*Xenopus* CDC6 and RPA antibodies were obtained from Dr Johannes Walter (Harvard Medical School); and rabbit anti-*Xenopus* RCC1 antibody was obtained from Dr Hideo Nishitani (Kyushu University, Japan); Mouse anti-PCNA monoclonal antibody (PC10) was purchased from Sigma and rabbit anti-*Xenopus* Polα p180, Polα p70, Polδ p125 and Polε p60 antibodies were obtained as described elsewhere (38). Histidine-tagged, N-terminal-truncated *Xenopus* geminin (10) was bacterially expressed and purified through nickel-charged Chelating Sepharose column chromatography (GE Healthcare Bio-Sciences). Glutathione *S*-transferase (GST)-fused p21 was bacterially expressed and purified through glutathione–Sepharose column chromatography (GE Healthcare Bio-Sciences).

Preparation and fractionation of egg extracts

Xenopus eggs were obtained from a chorionic gonadotrophin-injected female frog. The eggs were dejellied in 2% (w/v) cysteine and then rinsed three times in MMR (39) at room temperature. Dejellied eggs were activated by incubation in 0.5 μg/ml calcium ionophore A23187 (in MMR) for ~5 min at room temperature and then rinsed five times in ice-cold XB (39). Activated eggs were transferred to a SW55 tube (Beckman) which had previously contained 1 ml of ice-cold XB supplemented with 100 μg/ml of cytochalasin B and 10 μg/ml each of aprotinin, leupeptin and chymostatin, and then packed by centrifugation at 170 g for 1 min. After excess buffer was removed, cycloheximide was added at a final concentration of 0.1 mg/ml, and eggs were crushed by centrifugation at 13 500 g in a SW55Ti rotor (Beckman) for 15 min at 4°C (crushing spin). The cytoplasmic fraction was collected and supplemented with 0.25 mg/ml cycloheximide, and then cleared by re-centrifuging as above (clarifying spin). The cleared cytoplasmic fraction [referred to as the low speed supernatant (LSS)] was supplemented with 10 μg/ml each of cytochalasin B, aprotinin, leupeptin and chymostatin, and used immediately for experiments. In the experiment shown in Figure 4, clarifying spin was performed at 84 500 g for 15 min to partially remove nuclear membranes. The protein fraction, PEG-B, was prepared by fractionation of LSS as described elsewhere (40) and finally dialyzed against buffer LFB1/50 mM KCl (40). Immunodepletion of ORC in the egg extracts was achieved by treating the extracts twice (30 min each) with Protein A–Sepharose (GE Healthcare Bio-Sciences) coupled to anti-ORC1 and anti-ORC2 antibodies.

Replication of plasmid beads in egg extracts, and analyses of proteins and DNA bound to the beads

During the experimental procedure, special care has to be taken to avoid any loss of plasmid beads. In particular, protein-bound plasmid beads such as those formed after

incubation with egg extracts cannot be mixed or withdrawn by using a micropipette tip. Typically, 40 μg of plasmid beads (equivalent to ~240 ng plasmid in 4 μl) was washed once in 100 μl of ice-cold XB supplemented with 0.002% NP-40 (32). The beads were then mixed with 25 μl of egg extracts supplemented with 0.002% NP-40, ATP-regenerating system (20 mM creatine phosphate, 2 mM ATP and 6.3 μg/ml creatine phosphokinase) and 0.1 μl (37 kBq) of [α-^{32}P]dATP (110 TBq/mmol; GE Healthcare Bio-Sciences), and incubated for the indicated time periods at 23°C with occasional gentle agitation. After incubation, the reaction mixture was diluted with 150 μl of ice-cold XB supplemented with 0.002% NP-40, the beads were immediately separated using a magnet and as much solution as possible was then removed. The beads were washed three times with 100 μl each of ice-cold XB with 0.002% NP-40. For western blot analysis of proteins, the beads were resuspended in SDS sample buffer and treated at 100°C for 3 min before SDS–PAGE. After electrophoresis, proteins were transferred on to a nitrocellulose membrane and incubated with the appropriate antibodies. Detection for western blotting was performed using a Chemi-Lumi One detection kit (Nacalai Tesque, Inc., Kyoto, Japan).

For the analysis of DNA, the DNA was released from plasmid beads by cleaving the disulfide bond within the biotin-linker arm. After incubation with egg extracts, the reaction mixture was diluted and the beads were washed as above. The beads were then incubated in 100 μl of 25 mM Tris–HCl (pH 7.5), 7 mM EDTA, 0.7% SDS, 50 mM dithiothreitol and 1 mg/ml of freshly made proteinase K for 2 h at 37°C. The released DNA was then separated from the beads by using a magnet, extracted once with phenol–chloroform and precipitated with ethanol. DNA was separated by 0.8%

agarose gel electrophoresis (Tris/acetate/EDTA buffer) and after electrophoresis the gel was fixed in 10% methanol, 10% acetic acid for 30 min, dried on Whatman 3M paper and then exposed to an imaging plate for analysis using a Fuji image analyzer (BAS2500). The radioactivity of replication products was quantified with the accompanying Multi Gauge software (Version 3.0, Fujifilm) and the amounts of synthesized DNA were calculated, assuming that the concentration of dATP in LSS is 50 μM (41).

RESULTS

Incubation of a linear form of plasmid DNA immobilized to superparamagnetic beads in *Xenopus* egg extracts (LSS) leads to the beads being surrounded by nuclear membranes and the occurrence of DNA synthesis (31,32). However, the details of DNA replication under these conditions have not been investigated. Thus, we first examined whether DNA synthesis on an immobilized linear plasmid is inhibited by geminin, which is known to inhibit loading of MCM2–7 proteins on to DNA (10). In parallel, a supercoiled circular plasmid template was also coupled to the beads, using random biotinylation and coupling to streptavidin-magnetic beads (see Materials and Methods for details). We confirmed that supercoiling was maintained even after coupling of the DNA to the beads (Figure 1A).

As shown in Figure 1B, DNA synthesis on linear plasmid-coupled beads in LSS was unaffected by the addition of geminin (lanes 1–4), whereas DNA replication with sperm chromatin was almost completely inhibited at the same concentration of geminin (data not shown). The major DNA product with the linear plasmid beads was almost twice as long
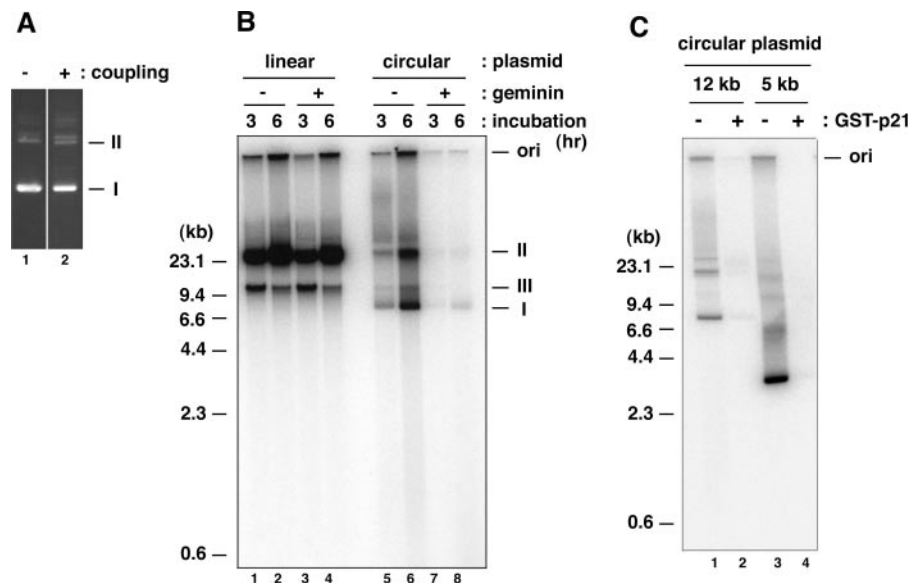

Figure 1. DNA synthesis on plasmid immobilized on paramagnetic beads in *Xenopus* egg extracts. (**A**) A circular form of plasmid pBluescript SK used for coupling to the beads (lane 1) or recovered from the immobilized beads (lane 2) was separated by agarose gel electrophoresis and stained with ethidium bromide. (**B**) A linear form (lanes 1–4) or circular form (lanes 5–8) of pG5λ6.6 (11 kb) immobilized on the beads was incubated in LSS in the presence or absence of 2 μg/ml geminin for the indicated time periods. (**C**) A circular form of pEXλ6.6 (12 kb) (lanes 1 and 2) or pKS-EX (5 kb) (lanes 3 and 4) immobilized on the beads was incubated in LSS for 4 h in the presence or absence of 13 μg/ml GST-p21. The ^{32}P-labeled replication products were separated by agarose gel electrophoresis and detected by autoradiography. Note that the major 23 kb product seen in lanes 1–4 in (B) migrated slightly faster than a nicked circular form of the plasmid. I, II and III denote fully supercoiled circular, nicked circular and linear forms of plasmid, respectively.

as the template size (Figure 1B, lanes 1–4). In contrast, DNA synthesis was severely inhibited by geminin when circular plasmid beads were used (Figure 1B, lanes 5–8). Supercoiled and nicked circular DNA was clearly detected in ^{32}P-labeled products (Figure 1B, lanes 5 and 6), indicating that complete DNA synthesis occurs on immobilized circular plasmids in a geminin-sensitive manner.

In addition to geminin, a CDK inhibitor, p21, inhibited DNA synthesis on immobilized circular plasmids, but not on linearized plasmids (Figure 1C and data not shown). DNA replication with sperm chromatin was also inhibited at the same concentration of p21 (data not shown). Therefore, DNA synthesis on an immobilized circular plasmid in LSS is initiated in a pre-RC- and CDK-dependent manner: however, since the exact way in which DNA synthesis is initiated on the linear plasmid is unclear, we chose to use circular plasmids in subsequent experiments.

A time course showed that only a background level of DNA synthesis occurred during the first 2 h of incubation, but DNA synthesis then increased markedly (Figure 2). The length of the time lag varied among the extracts (∼2–3 h). Only a background level of DNA synthesis was detected if circular plasmid-coupled beads were incubated in extracts depleted of nuclear membranes [so-called high-speed supernatant (HSS)], even after a prolonged incubation (data not shown). Furthermore, DNA synthesis on plasmid beads was severely inhibited when nuclear membranes in LSS were partially removed by centrifugation (84 500 g, 15 min, see Materials and Methods), whereas DNA replication with sperm chromatin was unaffected in the same partially depleted extracts (data not shown, also see Figure 4). Thus, the concentration of nuclear membranes in LSS may be more critical for DNA replication on plasmid beads, compared with replication on sperm chromatin.

Analyses of BrdU-labeled replication products by CsCl equilibrium density-gradient centrifugation showed that DNA is synthesized on circular-plasmid beads in a semi-conservative manner (data not shown). Aphidicolin, an inhibitor of replicative DNA polymerases, Polα, Polδ and Polε, inhibited DNA synthesis on circular-plasmid beads as efficiently as with the sperm chromatin template (data not shown). Therefore, bona fide DNA replication probably takes place on an immobilized circular plasmid in egg extracts. Owing to the difficulty in recovering DNA, particularly after prolonged incubation in the extracts, we were unable to quantify the precise amount of DNA synthesized on the immobilized plasmid; however, we estimate that ∼1–5% of the input DNA can be replicated.

The obvious advantage of the use of plasmid-coupled paramagnetic beads is that protein–DNA complexes formed on the plasmid can be isolated from the reaction mixture easily and quickly. We next analyzed the proteins that bound to plasmid beads during incubation with LSS, using western blotting. As shown previously (36), ORC and MCM3 were loaded on to circular plasmids on beads during the first 30 min of incubation (Figure 3A, lane 1, also see Figure 3B, lane 2). MCM loading on to the plasmid beads was not detected in ORC-depleted extracts, but addition of the ORC-containing fraction (PEG-B) (42) to the depleted extracts restored the loading of MCM proteins (Figure 3A, lanes 2 and 3). In addition, MCM loading on to circular
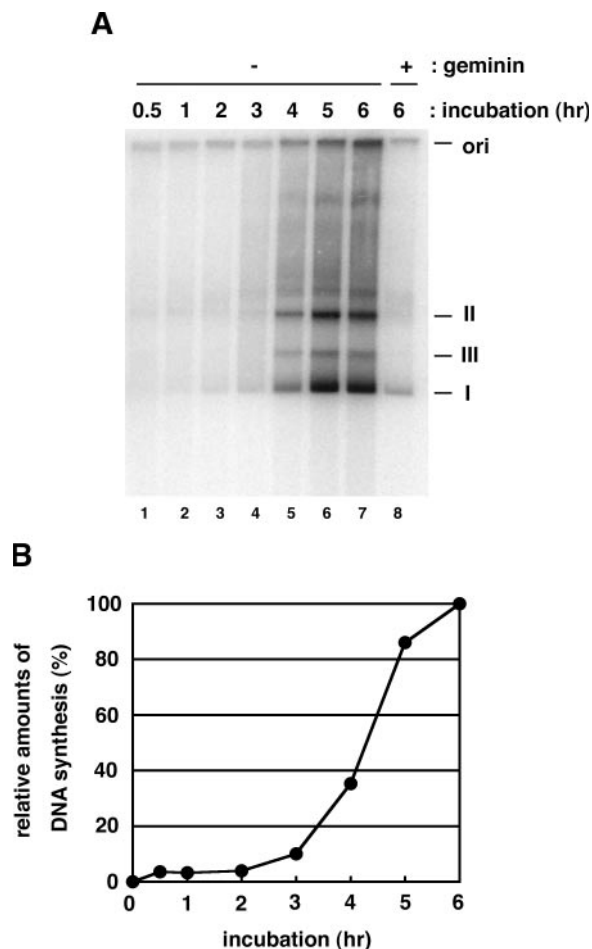

Figure 2. A time course analysis of DNA synthesis on circular plasmid beads in *Xenopus* egg extracts. A circular form of pG5λ6.6 immobilized on the beads was incubated in LSS for the indicated time periods. The reaction in lane 8 was performed using the extracts supplemented with 2 µg/ml geminin. The autoradiogram of the replication products (**A**) and the amounts of DNA synthesis relative to a 6 h incubation (**B**) are shown. I, II, and III in (**A**) denote fully supercoiled circular, nicked circular and linear forms of plasmid, respectively.

plasmid beads was inhibited by geminin, as shown previously (36), whereas loading was not affected by p21 (Figure 3B). Therefore, true pre-RCs appear to be formed on an immobilized circular plasmid. ORC1 and ORC2 were slightly displaced after pre-RC formation, whereas the level of bound MCM3 remained unchanged (Figure 3B and data not shown). RCC1 also became increasingly bound to plasmid beads during incubation, and since RCC1 is known to bind to chromatin, this observation generally reflects increased chromatin formation (Figure 3B).

In contrast to pre-RC formation and RCC1 binding, the binding of replication fork proteins (Polα, CDC45, RPA and PCNA) to plasmid beads was delayed, and only began after 2–3 h of incubation. Importantly, binding of these proteins to plasmid beads correlated with the start of DNA synthesis (see Figure 2 and data not shown). However, although DNA synthesis nearly reached a plateau after incubation in LSS for 5–6 h (data not shown and see Figure 2B), the levels of Polα, RPA and PCNA bound to the beads remained high until 6 h (Figure 3). This discrepancy may be due to
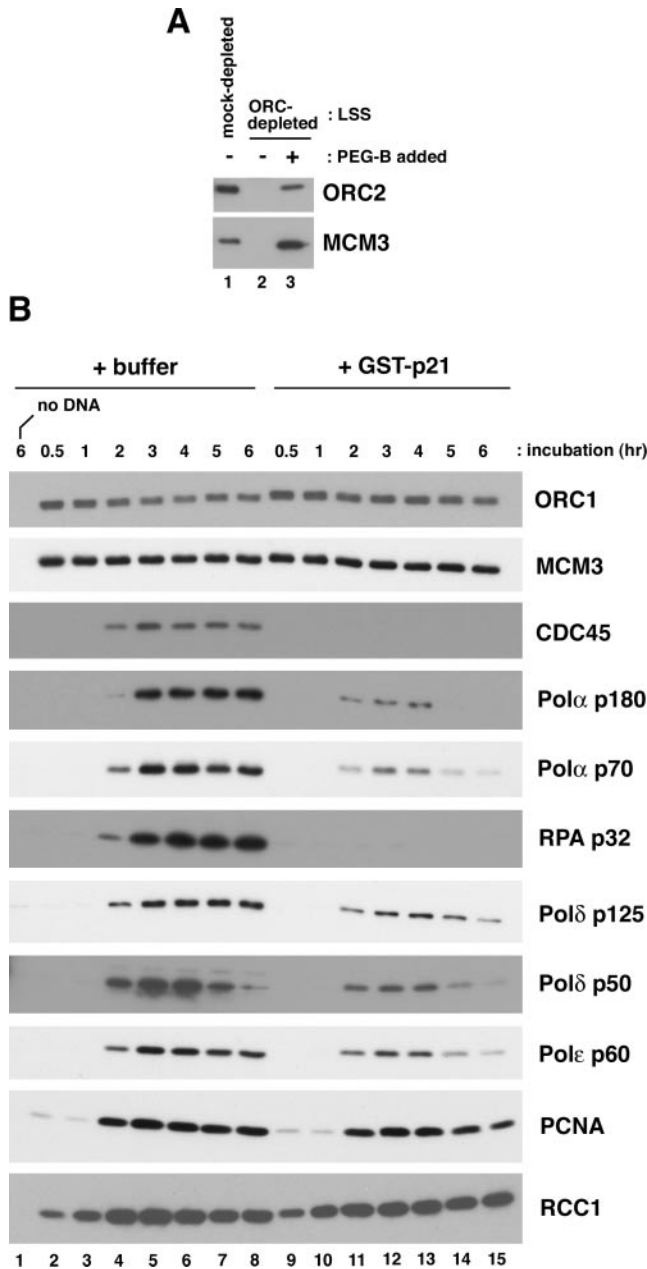
Figure 3. Analysis of the proteins bound to plasmid beads during incubation in *Xenopus* egg extracts. (**A**) pBluescript-coupled beads were incubated for 30 min in the mock-depleted (lane 1), the ORC-depleted extracts (lanes 2) or the ORC-depleted extracts supplemented with PEG-B fraction (lane 3). (**B**) pG5λ6.6-coupled beads or the beads alone (no DNA) were incubated in LSS for the indicated time periods in the absence or presence of 13 µg/ml GST-p21. After incubation, the proteins bound to the beads were analyzed by western blotting with the appropriate antibodies as indicated.

replication fork stalling at biotin–streptavidin links on the plasmid beads, causing replication fork proteins to remain bound to DNA without the occurrence of DNA synthesis. Interestingly, both Polδ and Polε, in addition to Polα, also bound to plasmid beads with kinetics similar to PCNA (Figure 3B), suggesting that all three DNA polymerases are involved in plasmid replication in egg extracts. More importantly, p21 significantly suppressed the binding of CDC45,
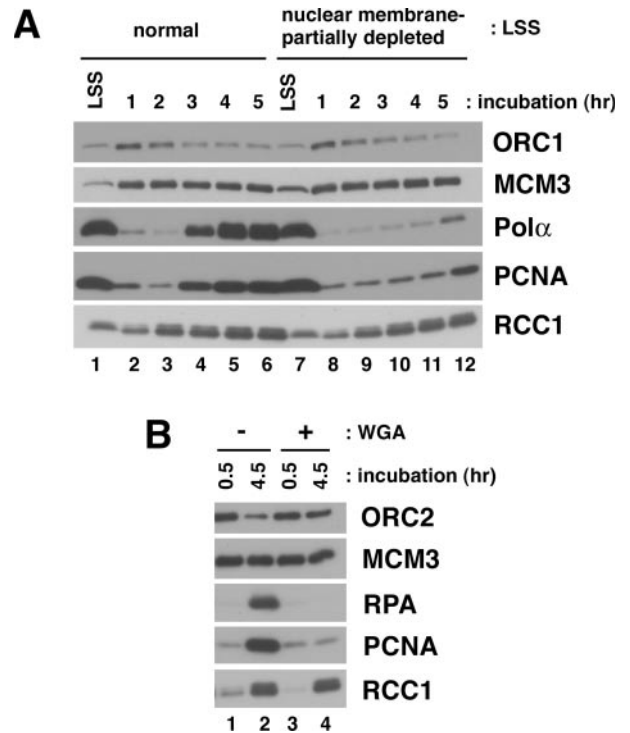

Figure 4. Requirement of nuclear formation for the binding of the replication fork proteins to plasmid beads. (**A**) pG5λ6.6-coupled beads were incubated in LSS prepared by the normal procedure (lanes 2–6) or partially depleted of nuclear membranes (lanes 8–12) for the indicated time periods. (**B**) pG5λ6.6-coupled beads were incubated in LSS supplemented with WGA (lanes 3 and 4) or the control buffer (lanes 1 and 2) for the indicated time periods. After incubation, the proteins bound to the beads were analyzed by western blotting with the appropriate antibodies as indicated. One microliter each of the extracts was loaded in lanes 1 and 7 in (A).

Polα and RPA to the plasmid beads, and also somewhat suppressed binding of Polδ, Polε and PCNA to the beads.

Consistent with the nuclear structure formation-dependent DNA replication suggested above, increased binding of replication fork proteins to plasmid beads did not occur when LSS was partially depleted of nuclear membranes, whereas there was little effect on the binding of ORC1, MCM3 and RCC1 under these conditions (Figure 4A). Furthermore, addition of WGA, a nuclear transport inhibitor, to the extracts also suppressed the increase in DNA binding of the replication fork proteins (RPA and PCNA), but not that of pre-RC components or RCC1 (Figure 4B).

Although geminin did not inhibit DNA synthesis on linearized plasmid-coupled beads, as shown above (Figure 1), loading of MCM proteins on to linear plasmids did occur and was inhibited by geminin, as shown in Figure 5 and previously (35). Curiously, the levels of MCM proteins loaded on to circular plasmids were higher than those loaded on to the same linearized plasmids, whereas ORC1 and CDC6 appeared to bind to both plasmid forms about equally (Figure 5A and B). However, when the pre-RC was first formed on circular plasmid beads, isolated and then treated with a restriction enzyme to linearize the DNA, the level of MCM proteins loaded on to the DNA was essentially unchanged after linearization (Figure 5C and D). These results suggest that DNA
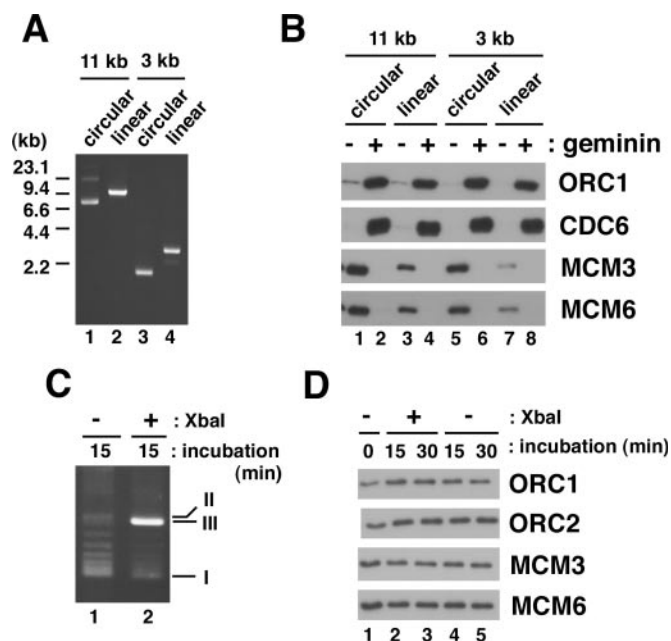
Figure 5. The effect of linearization of plasmid on pre-RC formation. (**A**) pG5λ6.6 (11 kb) or pBluescript (3 kb) coupled beads were pre-treated with XbaI to linearize the plasmid (lanes 2 and 4) or with the control buffer (lanes 1 and 3). After treatment, the plasmid was recovered from the beads, separated by agarose gel electrophoresis and stained with ethidium bromide. (**B**) The same pre-treated plasmid beads as in (A) were incubated in LSS supplemented with 2 µg/ml geminin (lanes 2, 4, 6 and 8) or the control buffer (lanes 1, 3, 5 and 7) for 30 min. The proteins bound to the beads were then analyzed by western blotting. (**C** and **D**) pBluescript-coupled beads were incubated in LSS for 30 min. The beads were then washed and treated with XbaI or the control buffer for 15 or 30 min. The plasmid after 15 min treatment with XbaI (C) and the proteins that remained bound to the beads (D) are shown. Lane 1 in (D) corresponds to the beads after 30 min incubation in LSS.

topology may affect the formation but not the stability of the pre-RC.

DISCUSSION

The data in this study show that DNA synthesis on linear plasmid-coupled beads takes place in egg extracts, even under conditions where loading of MCM proteins on to DNA is inhibited. Although it cannot be formally excluded that MCM proteins may still be involved in DNA synthesis on linear DNA, it seems likely that this synthesis is mediated by other nuclear activities, such as DNA repair. In contrast with the data for linearized plasmids, several lines of evidence suggest that genuine DNA replication takes place on immobilized circular plasmids in the egg extracts. First, MCM3 and MCM6, probably as the MCM2–7 protein complex, were loaded on to plasmid beads in an ORC-dependent manner, and this MCM loading was inhibited by geminin. Second, DNA synthesis occurred in a semi-conservative manner and was also inhibited by geminin and p21, indicating that pre-RC formation and CDK activity-dependent DNA replication occurs. Third, the start of DNA synthesis correlated with the DNA binding of replication fork proteins such as CDC45, RPA, DNA polymerases and PCNA, and binding of these proteins was inhibited by p21. Finally, both binding of replication fork

proteins and DNA synthesis were dependent on the formation of a functional nuclear structure.

Although the pre-RC can be formed efficiently on circular plasmid-coupled beads [as shown herein, and also see Ref. (36)], it is quite possible that the biotin–streptavidin link between the beads and the DNA affects certain processes during DNA replication, particularly in the elongation step. One can imagine that, if a replication fork encounters this link, it may stall immediately. Nevertheless, a closed circular form of replicated DNA was clearly produced, as shown in Figure 1. Although we could not determine the number of biotin–streptavidin links formed on each plasmid, some plasmids may have only one biotin–streptavidin link per plasmid. Under these circumstances, two replication forks could meet each other at the link, thereby allowing the DNA strand that is not directly connected to the bead to be replicated completely, and producing a closed circular daughter molecule. In contrast, if biotin–streptavidin links exist at multiple sites on one plasmid, replication cannot easily be completed and the plasmid may tend to remain bound to the bead, even after the treatment to induce link breakage. This may explain why it is so difficult to recover DNA from the plasmid beads, particularly after prolonged incubation in the egg extracts.

The efficiency of DNA synthesis under the conditions described here is relatively low. Consistent with this, the levels of MCM proteins loaded on to DNA did not decrease during DNA synthesis (Figure 3B). However, it should be noted that, in addition to Polα and Polδ, Polε binds to plasmid-coupled beads during DNA synthesis (Figure 3B). This implies that Polε, as well as Polδ, may be a component of the replication fork and may be required for plasmid replication. If so, this plasmid replication system may allow elucidation of the role of Polε at the replication fork, which has not been possible to determine in previous studies using *in vitro* SV40 DNA replication (43). Consistent with our results, a recent study has shown enrichment of Polα, Polδ and Polε at the replication barrier on a plasmid in *Xenopus* egg extracts (44). Although we have prepared Polε-immunodepleted egg extracts and used them in the plasmid replication system, clear and reproducible results are yet to be obtained, because DNA synthesis activity in the extracts decreases dramatically after depletion treatment, even with mock depletion.

A nuclear-free *Xenopus* DNA replication system in which free plasmid can be replicated synchronously and very efficiently has been reported previously (26). Our preliminary data for quantification of PCNA bound to plasmid beads suggest that the pre-RC may be activated and the replication fork may eventually be formed on most of the immobilized plasmids under the conditions described here. However, in contrast with the nuclear-free system, pre-RC activation on plasmid beads in LSS occurs asynchronously over a long period of time. This is probably because functional nuclear structure may not be formed efficiently on the plasmid beads. Alternatively, pre-RC activating proteins such as cyclin E may not accumulate efficiently within the nucleus in the presence of the paramagnetic beads.

Despite relatively low DNA synthesis activity, the plasmid replication system described here should still be useful for studying eukaryotic DNA replication and particularly for analyzing replication proteins bound to a plasmid template.

The use of magnetic beads allows rapid and simple isolation, and analysis of protein–DNA complexes on the plasmid-coupled beads. Moreover, because of the rapid timeframe, isolation of relatively unstable complexes may also be possible using this system and, in fact, we have successfully isolated such complexes using circular plasmid beads (36). We believe that the circular plasmid-coupled beads can also be applied for studies using extracts prepared from mammalian cells.

Another feature of the replication system is that the supercoiled state in circular DNA is changeable during incubation, despite the attachment of the plasmid to the beads. It is known that DNA topology can affect many activities occurring on DNA. In fact, our data indicate that MCM proteins can be loaded on to circular DNA more efficiently than onto linear DNA, although ORC and CDC6 seem to bind to both forms of DNA about equally. This latter observation is somewhat different from the previous finding that purified *Drosophila* ORC binds preferentially to supercoiled DNA (45). This discrepancy may be due to different conditions for ORC binding in these experiments; i.e. a large excess of ORC was used in egg extracts under our conditions. Nevertheless, our data suggest that DNA topology may also affect the MCM loading step, in addition to DNA binding of ORC.

In addition to pre-RC formation, nucleosomes are assembled on plasmid beads in egg extracts (A. Zembutsu and S.Waga, unpublished data) and there may be competition among nucleosome assembly, ORC binding and MCM loading on to DNA. Thus, it will be interesting to see whether the nucleosome itself plays any role in the formation of the pre-RC as suggested previously (46). This specific question can be answered using the circular-plasmid replication system presented here.

ACKNOWLEDGEMENTS

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