DNA Replication in Protein Extracts from Human Cells Requires ORC and Mcm Proteins*

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We used protein extracts from proliferating human HeLa cells to support plasmid DNA replication in vitro. An extract with soluble nuclear proteins contains the major replicative chain elongation functions, whereas a high salt extract from isolated nuclei contains the proteins for initiation. Among the initiator proteins active in vitro are the origin recognition complex (ORC) and Mcm proteins. Recombinant Orc1 protein stimulates in vitro replication presumably in place of endogenous Orc1 that is known to be present in suboptimal amounts in HeLa cell nuclei. Partially purified endogenous ORC, but not recombinant ORC, is able to rescue immunodepleted nuclear extracts. Plasmid replication in the in vitro replication system is slow and of limited efficiency but robust enough to serve as a basis to investigate the formation of functional pre-replication complexes under biochemically defined conditions.

The assembly of pre-replication complexes (pre-RC) on mammalian genomes has been well described. As in other eukaryotes, it begins with the binding of an origin recognition complex (ORC) to chromatin. ORC consists of a stable core complex including subunits Orc2-Orc5 associated with less stably bound subunits Orc1 and Orc6 (1–4). Chromatin-bound ORC recruits two other proteins, Cdc6 and Cdt1, of which Cdc6 stabilizes the binding of ORC (5) and allows Cdt1 to load the hexameric Mcm complex (composed of Mcm2-Mcm7) (reviews in Refs. 6–9).

At the beginning of S-phase, pre-RCs are converted into active replication forks under the guidance of protein kinases Cdc7 and Cdk2. This process requires the origin binding of several additional initiation factors, including Mcm10 and Cdc45, which together initiate a series of events that ultimately lead to origin unwinding, recruitment of replicative DNA polymerases, and the establishment of replication forks (6).

Although the order of the reactions appears to be well established, many mechanistic details have yet to be worked out, and for that purpose it would be useful to possess an in vitro replication system with completely soluble proteins and a biochemically amenable DNA template. A highly successful replication system with soluble constituents is based on extracts from Xenopus eggs. The key components are a membrane-free cytoplasmic extract (for the chain-elongating functions) and a highly concentrated nuclear extract (for initiator proteins) (10). The system replicates either frog sperm chromatin or protein-free plasmid DNA and has been and still is very successful in investigations into the events leading to pre-RC formation (11–15) and to characterize important replication factors such as Cdt1 and Mcm8 (16, 17). The Xenopus egg extract contains high amounts of initiator proteins such as ORC and Mcm proteins, stored for subsequent rounds of cell divisions in early embryogenesis. It therefore differs from the situation in proliferating adult mammalian cells with their limited amounts of ORC.

In vitro replication systems with cytosolic mammalian cell extracts, using simian virus 40 (SV40) DNA as template, and a viral protein, T antigen, as initiator have been used since the mid-1980s (18–21) and have been extremely successful in identifying many of the mammalian proteins required for replicative chain elongation (reviews in Refs. 22, 23). In this system, the cytosolic cell extract is the source for the main chain-elongating functions, whereas SV40 T antigen is the single initiator protein that targets a unique origin site in the viral genome where it unwinds the DNA double strand and then functions as a helicase at the emerging replication forks (review in Ref. 24).

Much research has been done to establish in vitro replication systems entirely dependent on soluble proteins from proliferating mammalian cells in culture (25–27). Optimal DNA synthesis in these systems requires a cytosolic extract and a high salt nuclear extract and allows semiconservative replication of plasmid DNA (with eukaryotic DNA inserts), although to an extent that is only 1–3% of that achieved with T antigen and SV40 DNA under similar biochemical conditions. This is quite plausible because the initiator T antigen is added in high amounts to the incubation mixtures and does not need to interact with other proteins for the recognition and the unwinding of the viral origin. In contrast, the dozen or so cellular initiator functions are inevitably diluted when extracted from the nuclei of cultured cells and assemble on one of many possible sites on the plasmid template where they must interact in a highly complex manner before they are ready to initiate a replication round.

With these limitations in mind we decided to investigate whether ORC and other components of the pre-RC are necessary for replication initiation in vitro. We used an in vitro system with proteins extracted from proliferating HeLa cells. The template was a plasmid, pEPI-1, that replicates as an autonomous unit (“episomally”) in mammalian cells and has no specific origin and ORC binding site in vivo (28). We performed immunodepletions and showed that Orc proteins and Mcm proteins are indeed active components in a high salt nuclear extract. Endogenous ORC, but not recombinant ORC, is able to complement a nuclear extract immunodepleted of Orc proteins. The main conclusion was that the in vitro system appeared to be sufficiently robust to investigate questions related to replication initiation in mammalian cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Synchronization—HeLa S3 cells were grown to semiconfluency on plastic dishes in Dulbecco’s modified Eagle’s...
medium plus 5% fetal calf serum. For synchronization, cells were first subjected to a double thymidine block (29) and then released for 2 h (for cells in S-phase) or for 15.5 h (for cells in G1 phase). Cell cycle phases were verified by flow cytometry (not shown).

Preparation of Cell Extracts—Soluble proteins were prepared according to Gruss (30). Briefly, HeLa cells on plastic dishes were washed twice in cold phosphate-buffered saline, collected in buffer A (20 mM HEPES, pH 7.4, 5 mM KCl, 1.5 mM MgCl2, 0.1 mM dithiothreitol) containing 250 mM sucrose, and washed twice with buffer A. They were then kept for 10 min on ice in a small volume of buffer A and disrupted in a type S Dounce homogenizer. Nuclei were collected by centrifugation at 16,000 × g for 10 min. The supernatant was then centrifuged at 100,000 × g for 1 h to prepare the S100 extract (10–15 μg of protein/μl), which was stored in aliquots at −70 °C. The nuclear pellet was washed in buffer A and then kept in buffer A containing 450 mM potassium acetate for 90 min on ice. After preclearing (12,000 × g for 5 min), the supernatant was centrifuged at 300,000 × g for 1 h to prepare the S300 extract (3–8 μg of protein/μl), which was stored in aliquots at −70 °C.

Cell and chromatin fractionations were performed as described (29). Immunoprecipitations with affinity-purified antibodies against human Orc1, Orc2 (29), and Mcm3 protein were performed as previously described (31). Control antibodies were nonspecific rabbit immunoglobulin G (IgG) from Sigma.

In Vitro Replication—As templates we used plasmids pEPI-1 and its derivatives pEPI-UPR and pEPI-EX9 (28) purified by CsCl equilibrium centrifugation. Preparation and use of T antigen were exactly as described previously (30). We performed preincubations in a total volume of 35 μl with S300 nuclear extract (16 μg) and pEPI-1 DNA (160 ng) at 2 mM ATP and 80 mM potassium acetate in buffer A plus 1 μl of Complete, EDTA-free (Roche Applied Science), as protease inhibitor. Replication was initiated by the addition of the S100 extract (45 μg), 40 mM creatine phosphate, 30 mM potassium acetate, 80 μM CTP, GTP, and UTP, 100 μM dGTP, dCTP, and dTTP, 30 μM dATP, and 10 μCi of [α-32P]dATP (ICN) in a total volume of 50 μl. Incubation was at 37 °C for 60 min (if not stated otherwise) and then stopped by 30 μl of stop mix (60 mM EDTA, 2% SDS). DNA was extracted by proteinase K digestion and phenol-chloroform treatment followed by ethanol precipitation.

Recombinant ORC—Open reading frames of human Orc1, Orc4, and Orc6 were cloned into the multiple cloning site of pBlueBacHis2 (Invitrogen) to generate amino terminal His-tagged proteins. Human Orc2, Orc3, and Orc5 were cloned into pVL (Invitrogen). SF-9 insect cells were cotransfected with linearized Autographa californica nuclear polyhedrosis virus baculovirus DNA and the Orc clones according to the Invitrogen protocol. High titer virus stocks were raised from single plaque isolates.

For the expression of ORC, High-Five insect cells were coinfected with all six recombinant baculoviruses at a multiplicity of infection of 6. The cells were harvested after 3 days and the recombinant proteins purified as described by Vashee and co-workers (32). The eluate from nickel-agarose was further purified by gel retardation gradient centrifugation (10–30% glycerol) in buffer OBB2 (50 mM HEPES, pH 7.4, 5 mM MgCl2, 0.1 mM EDTA, 0.1 mM EGTA, 0.01% Tween 20, 500 mM NaCl, 10 mM mercaptoethanol). Centrifugation was at 45,000 rotations/min for 16 h at 4 °C in the Beckman SW55 rotor. Fractions with Orc were stored in aliquots at −70 °C. For some experiments, His-Orc1 was dephosphorylated with 400 units of A-phosphatase/10 pmol of recombinant Orc1 in accordance with the manufacturer’s protocol (New England Biolabs) for 90 min at 30 °C.

DNA Binding—For nicked circling DNA binding assays, pEPI-1 was linearized by BamHI restriction and end labeled with [α-32P]dATP using the Klenow fragment of DNA polymerase I. The 400-μl binding reaction contained 50 ng of labeled DNA in OPB2F-buffer (50 mM HEPES, pH 7.4, 5 mM MgCl2, 0.1 mM EDTA, 0.1 mM EGTA, 0.01% Tween 20, 80 mM NaCl, 10 mM mercaptoethanol, 100 ng/ml of bovine serum albumin, and 1 mM ATP where indicated) and different amounts of recombinant ORC. After incubation at 25 °C for 30 min, the mixture was passed through nitrocellulose filters (BA45; Schleicher and Schuell), washed with OPB2, dried, and quantitated in a scintillation counter.

The principle of the target-bound DNA binding assay has been described by Hinzpeter and co-workers (33). Briefly, pEPI-1 was

FIGURE 1. In vitro replication requires soluble and chromatin-bound nuclear proteins. A, map of pEPI-1. The human scaffold/matrix attached region (S/MAR) was cloned into the multiple cloning site of pUC19 (C) containing the SV40 origin was replicated by a cell-free system consisting of a soluble protein extract (S) of HeLa cells supplemented with large quantities of large T antigen (T-Ag) for 1 h. Deproteinized replication products were digested by Dpn1 and analyzed by agarose gel electrophoresis and autoradiography (30). HMW, high molecular-weight DNA; R, relaxed and open circular DNA; I, superhelical form I DNA. C, 160 ng of pEPI-1DNA containing the SV40 origin was replicated by a cell-free system consisting of a soluble (S) and/or chromatin-bound nuclear protein (N) extract for 1 h. I, linearized DNA. D, soluble (S) and salt-extracted nuclear (N) protein extracts were analyzed by Western blotting with Orc1-, Orc2-, or Mcm3-specific antibodies.

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digested with NcoI, BglII, and BamHI to create fragments that were end labeled as just described. Recombinant SV40 large T antigen and native or recombinant Orc proteins and complexes were immunoprecipitated with monoclonal or monospecific antibodies and protein A-Sepharose. 100 µl of binding buffer containing 50 ng of labeled DNA, 80 mM NaCl, 1 mM ATP, 6 µg/ml of cytosolic extract, and increasing amounts of herring sperm DNA as competitor were added to the beads and incubated for 30 min on ice. After a short centrifugation, beads were washed twice with binding buffer and protein-DNA complexes were eluted in Laemmli buffer (34), separated by polyacrylamide gel electrophoresis, and analyzed by autoradiography and Western blotting.

RESULTS

A Nuclear Extract Is Required for Plasmid Replication in Vitro—The template used in these studies is the plasmid pEPI-1 (Fig. 1A), which is known to replicate semiconservatively as an independent unit in a one-per-cell cycle manner in several mammalian cell lines in vivo (28). The sequence elements, essential for episomal replication and mitotic segregation, are the strong cytomegalovirus promoter and a matrix attachment region. The plasmid also contains the SV40 origin which is, however, dispensable for replication in vivo (35) but convenient for the in vitro studies reported below because it facilitates control experiments with the SV40 initiator protein, T antigen.

Accordingly, pEPI-1 was incubated with T antigen and a soluble S100 extract under in vitro replication conditions. It yielded replication products of various topological forms of circular DNA as well as a broad spectrum of high molecular weight replicative intermediates as previously described (30) (Fig. 1B). Most replication products were resistant to DpnI, which only cleaves DNA that is methylated on both strands (as the parental pEPI-1 that had been produced in bacteria), indicating that the DNA was either hemimethylated or unmethylated and had therefore undergone at least one round of replication in vitro. The controls here were incubations with T antigen alone and with the S100 extract alone. The latter incubation yielded some labeled DNA that was, however, degraded by DpnI and therefore was due to repair DNA synthesis (Fig. 1B). The results were in agreement with earlier studies and demonstrated that T antigen is an efficient initiator and that the soluble S100 extract contains all the major functions required for replicative chain elongation.

Next, we replaced T antigen by a nuclear extract (S300 extract) from HeLa cells as a potential source for initiator proteins and performed in vitro incubations under the same conditions as in Fig. 1B. We obtained DpnI-resistant replication products with the electrophoretic properties of open circular form II and linear form III DNA (Fig. 1C). The replication products were resistant to DpnI and also to MboI (not shown), which cleaves only unmethylated DNA, indicating that the labeled DNA was the product of one round of replication. The amount of label incorporated into DpnI-resistant DNA was only 1–2% of that incorporated in the T antigen-dependent reaction. Therefore, to visualize the replication products, longer exposure times were necessary with the conse-
quence that the DpnI-sensitive products of repair synthesis are more prominent in Fig. 1C than in Fig. 1B. However, the main conclusion from Fig. 1C is that the S300 nuclear extract contained important replication-initiating functions. These could include Orc proteins because Orc1 and Orc2 were present in the nuclear extract but not in the soluble S100 extract (Fig. 1D). Mcm3 and other Mcm proteins (not shown, but see Ref. 31) were also present in the nuclear extract. An equal fraction of Mcm3 additionally appeared in the S100 extract of soluble proteins (Fig. 1D). This was expected because it is known that during all cell cycle phases a considerable fraction of Mcm proteins is free in the nucleus and not bound to chromatin (31).

We have performed many experiments to characterize the replication system. In Fig. 2, we present some of the results. A first and important point is that replication in vitro was optimal when both the soluble S100 and the nuclear S300 extract were from S-phase cells. In contrast, almost no replication was observed when both extracts were from G1 phase cells. Interestingly, it was more important that the nuclear extract, rather than the soluble extract, was from S-phase cells (Fig. 2A), indicating that a crucial component of the initiating activity in the nuclear extract must be modified during the G1 to S-phase transition. The nature of this component and its modification are presently not known. We also found that the supercoiled form of pEPI-1 was the preferred template and that the open circular form II DNA and the linear form III DNA functioned poorly or not at all as templates (not shown).

Furthermore, DNA synthesis was absolutely dependent on ATP and an ATP-regenerating system (not shown) (27). It was sensitive to aphidicolin (Fig. 2B) and insensitive to dideoxy nucleotide triphosphates (not shown) (27), indicating that replicative DNA polymerases are involved in the in vitro replication reaction. We also found that α-amanitin and RNase H treatment had no effect on in vitro DNA replication (not shown), excluding the possibility that RNA polymerase-dependent RNA synthesis or residual RNA could provide the primers for the in vitro synthesis of DNA.

When the products of ongoing replication were investigated after denaturation on alkaline-agarose gels, we detected short labeled DNA strands (200–1000 bases) and larger DNA strands, which we interpret as lagging and leading strands. The shorter (lagging) strands were converted to unit-length units when replication ceased (Fig. 2C). Indeed, even under optimal conditions, DNA replication in vitro was slow and rather inefficient (Fig. 2D), replicating just one in a few hundred plasmid templates.

The limiting factor responsible for the inefficient reaction must be in the nuclear extract, because the soluble extract with the replicative elongation functions was highly efficient in T antigen–directed replication in vitro (see Fig. 1B). A plausible explanation is that the necessary initiator proteins such as ORC and Mcm proteins and others are present in dilute quantities in the nuclear extract. Consequently, the probability that the various components of pre-RC meet and assemble properly on pEPI-1 will be rather low. Below, we first show that Orc1 and Orc2 as well as Mcm3 are indeed required for replication initiation in vitro and then report on our attempts to rescue an immunodepleted nuclear extract by exogenous proteins.

**Orc1, Orc2, and Mcm3 Are Required for in Vitro Replication**—As shown in Fig. 1, Orc1 and Orc2 (and other Orc proteins, not shown) are present in the nuclear extract, as is that fraction of Mcm3 (and other Mcm proteins, not shown) that is bound to chromatin in replication-competent cells. We investigated whether these pre-RC proteins were needed for in vitro DNA replication and performed depletion experiments with affinity-purified antibodies (Fig. 3A). Nuclear extracts depleted of Orc1, Orc2, and Mcm3 were completely inactive in initiating in vitro DNA replication, whereas nuclear extracts treated in parallel experiments with unspecific antibodies were fully active (Fig. 3B). In nuclear extracts, Orc1 and Orc2 are components of multisubunit ORC subcomplexes (see below), just as Mcm3 is always a component of one of several subcomplexes with other Mcm proteins (6). We conclude therefore that ORC and Mcm proteins participate in replication initiation in vitro.

An interesting point here is that the S100 extract contains the soluble fraction of Mcm proteins that is not bound to chromatin (see Fig. 1D). However, this fraction of unbound Mcms is unable to substitute for the chromatin-bound Mcm3 in the nuclear extract and cannot induce DNA replication in vitro (Fig. 3).

**Recombinant ORC Fails to Complement Depleted Nuclear Extract**—We have tried to complement the depleted nuclear extracts with recombinant proteins. We prepared recombinant ORC by cotransfecting insect cells with six baculovirus clones expressing the six common ORC subunits. The resulting complex was extracted via the histidine tags of some of the subunits and further purified by glycerol gradient centrifugations. The five largest of the six subunits form a stable Orc1–5 subcomplex that sedimented with ~9 S as expected if each subunit were present in one copy each. The sixth subunit, Orc6, was not part of the complex but sedimented independently as a monomeric protein (Fig. 4A). This result agrees with the findings of others who have reported that Orc6 only weakly interacts with the Orc1–5 subcomplex (1–4).

Using a filter binding assay, we tested the DNA binding activity of the Orc1–5 subcomplex in the glycerol gradient and determined that it bound efficiently to DNA in the presence, but not in the absence, of ATP. A fraction enriched for Orc4 also bound to DNA although independently of ATP and with reduced efficiency (Fig. 4B). To detect a possible sequence specificity of DNA binding, we used labeled restriction fragments of pEPI-1 in the target-bound assay that determines the formation of protein-DNA complexes in immunoprecipitates. A control experiment is shown in Fig. 4C that demonstrates that, as expected, T antigen specifically bound to that plasmid DNA fragment that contains the SV40 origin. In contrast, the Orc1–5 subcomplex bound without specificity to all pEPI-1 fragments (Fig. 4D). Orc6 had no DNA binding activity of its own (Fig. 4A) but stimulated the DNA binding of...
Orc1–5 by a factor of 1.5–2 (not shown). Thus, the recombinant Orc proteinshad the expected properties as they formed a stable ORC subcomplex that bound to DNA in an ATP-dependent manner (32). However, recombinant Orc1 alone or the gradient-purified Orc1–5 subcomplex failed to complement the nuclear extract depleted by Orc1-specific antibodies (Fig. 4E). Addition of Orc6 did not change the result (not shown). Furthermore, because Orc proteins produced in insect cells carry a phosphorylation pattern different from that of Orc proteins in mammalian cells,7 we treated recombinant Orc proteins with λ-phosphatase but this had no effect on in vitro DNA replication (Fig. 4E). Similar complementation experiments were performed with nuclear extracts depleted by Orc2-specific antibodies, but the results were also negative (not shown).

One possibility is that the His tags or other modifications of the recombinant proteins Orc1, Orc4, and Orc6 interfere with their biochemical functions in vitro. To study this possibility, we added recombinant Orc1 to the standard replication assay, i.e. an assay with untreated nuclear extract. The rationale for this experiment is that endogenous Orc1 is known to be rapidly degraded in vitro (29, 36) with the possible consequence that the amounts of Orc1 in the S300 nuclear extracts were less than optimal for in vitro replication. Indeed, recombinant Orc1 added to the nuclear extract stimulated severalfold the standard replication reaction. Stimulation was greatest when recombinant Orc1 was dephosphorylated before use (Fig. 5) either because

7 C. Damoc and M. Baack, unpublished information.
phosphatase treatment removed inhibitory phosphate groups or because dephosphorylated Orc1 is more stable during the incubation in vitro.

Interestingly, addition of the recombinant Orc1–5 subcomplex, dephosphorylated or not, had no effect on replication in the standard in vitro assay. Thus, although isolated recombinant Orc1 appears to function well in in vitro replication, the recombinant Orc1–5 subcomplex has properties that prevent it from functioning in standard replication assays and in immunodepleted S300 nuclear extracts.

A possibility is that recombinant ORC requires a specific modification that endogenous ORC has or that immunodepletion of nuclear extracts removes an associated factor that is necessary for the formation of functional pre-RC. In either case, endogenous ORC as extracted from proliferating cells should be able to replace the immunoprecipitated activity in nuclear extracts.

Endogenous ORC Is Active in DNA Replication in Vitro—ORC is normally firmly bound to chromatin, and it needs high salt for extraction from nuclei or isolated chromatin. As previously described, an Orc2–5 subcomplex is eluted from HeLa cell chromatin at 250 mM NaCl, whereas 350 mM NaCl are necessary to mobilize the Orc1–5 subcomplex (29). (Fig. 6A). The immunoprecipitation experiment of Fig. 6B confirmed that essentially all Orc1 in the 350-mM eluate was associated with Orc2 and with subunits Orc3–Orc5 (not shown), forming the larger Orc1–5 subcomplex. As shown by target-bound assays, the Orc1–5 subcomplex bound well to DNA (Fig. 6C), perhaps with a slight preference for a DNA fragment that contained a previously mapped cellular origin (Fig. 6C, UPR, upstream promoter region) (37). More importantly in the present context, the 350-mM chromatin eluate with the Orc1–5 subcomplex and free Orc1, but not the 250-mM chromatin eluate with the Orc2–5 subcomplex, could complement the ORC-depleted nuclear fraction in the in vitro replication assay (Fig. 6D). For further characterization, we fractionated the 350-mM preparation by sucrose gradient centrifugation and determined a co-sedimentation of Orc1–5 and the complementing activity (Fig. 7) confirming that it is the Orc1–5 subcomplex in the S300 nuclear extract that complements the immunodepleted extract.

Complementation was observed with two different preparations of Orc1–5. In one preparation, HeLa cell chromatin was first treated with 250 mM NaCl to remove the Orc2–5 subcomplex and then with 350 mM NaCl for the preparation of the Orc1–5 complex (Fig. 6D, lane 4). In the

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**FIGURE 5. Dephosphorylated recombinant human Orc1 increases replication activity.** A, dephosphorylation by λ-phosphatase treatment. Recombinant human ORC (Orc1–5, fraction B of the glycerol gradient) or His-Orc1 were treated with λ-phosphatase (λ-PPase). Proteins before and after treatment were subjected to 10% SDS-PAGE analysis and stained with Coomassie Blue. B, effect of recombinant ORC on the in vitro replication activity. Untreated (2 and 4 μg) and λ-PPase-treated (4 μg) Orc1 protein or Orc1–5 were added to the standard in vitro replication system. Control assays without additional recombinant protein (lane C) and after addition of buffer (lane B) were performed.

**FIGURE 6. Endogenous human ORC binds DNA and is able to rescue a depleted nuclear extract.** A, cell fractionation. HeLa cells were used to prepare cytoplasmic proteins (Cy), nucleosolic proteins (Nu), and a residual nuclear structure (chromatin and nuclear matrix) that was successively washed with 250, 320, and 420 mM NaCl. The supernatants were investigated by Western blotting using Orc1- and Orc2-specific antibodies. P, pellet after salt washing steps. B, free Orc1 protein in the 250 mM extract. Nuclear extracts, prepared at 250 and 350 mM NaCl, were incubated with Orc1-specific or unspecific (IgG) antibodies as indicated. Immunoprecipitations were performed and processed for Western blotting. The blotted proteins were stained with a mixture of Orc1- and Orc2-specific antibodies. C, target-bound assay with fractionated endogenous protein extracts. Fractionated nuclear extracts were incubated with Orc1-specific or unspecific (IgG) antibodies as indicated and coupled to protein A-Sepharose. A 250-mM salt extract and a post-extracted 350-mM salt extract were investigated. Binding reaction was performed with 50 ng of labeled pEPI-1-DNA-fragments and an increasing amount of competitor. UPR indicates the fragment containing an authentic origin of DNA replication. D, rescue of depleted extracts by endogenous ORC. Before (lane 1) and after addition of fractionated endogenous protein extracts to Orc1-depleted nuclear extracts, pEPI-1 was replicated in vitro. A 250-mM salt extract, the corresponding post-extracted 350-mM salt extract, and a complete 350-mM salt extract were investigated. As control, nuclear extract incubated with IgG antibodies was used (lane C).
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A

kDa
4.3S
8.9S
17S

0
1
2
3
4
5
6
7
8
9
10
11

B

Replication

FIGURE 7. Whole ORC, and not free Orc1p, rescues an Orc1-depleted extract. A, sucrose gradient sedimentation of endogenous human cell extracts. Nuclear protein extracts were loaded onto a 10–50% sucrose gradient and centrifuged for 16 h at 45,000 rpm in a Beckman TL40 rotor at 4 °C. After collection of 11 fractions from the top to the bottom of the tubes, aliquots (20 μl) of each fraction were subjected to 10% SDS-PAGE analysis and processed for Western blotting. The blotted proteins were stained with Orc1- and Orc2-specific antibodies. B, rescue of depleted extracts by sucrose gradient fractions after sedimentation. After concentration of the collected fractions by centrifugation through Vivasin Concentrator, each fraction was added to Orc1-depleted nuclear extracts and pEPI-1 was replicated in vitro and investigated as described.

second preparation chromatin was directly treated with 350 mM NaCl, yielding an extract containing both the Orc2–5 subcomplex and the Orc1–5 subcomplex (Fig. 6D, lane 5). Apparently, Orc2–5 is not sufficient and not required for complementation.

DISCUSSION

Plasmid DNA is efficiently replicated in vitro when it contains the SV40 origin of replication and when the viral protein T antigen is used as an initiator. T antigen specifically binds to the origin and induces the unwinding of the DNA double strand. All major functions required for the establishment and propagation of replication forks are provided by a soluble protein extract from mammalian cells (22, 23). This highly successful system serves as a model to establish a replication assay that entirely depends on cellular mammalian proteins. The obvious choice is to replace T antigen by a protein extract prepared at high salt from isolated nuclei or chromatin. The choice is obvious because a high salt nuclear extract contains many of the proteins that are known or believed to be involved in the initiation of mammalian genome replication such as Orc and Mcm proteins, Cdt1 and Cdc6.

Indeed, previous experiments with soluble and nuclear extracts from human cells have shown that replication in vitro is semiconservative and depends on the known replicative DNA polymerases. The aim of most previous studies was to show that replication does not start randomly on the plasmid DNA sequences but prefers cloned cellular sites that correspond to replication start sites in vivo or to early replicating sequences (26, 27, 38–42).

In the present study, we took advantage of this in vitro replication system and used as template a plasmid, pEPI-1. This plasmid replicates as an extrachromosomal replicon in mammalian cells and faithfully segregates during mitoses in many cell generations. In vivo, pre-RCs assemble at many sites on the plasmid, perhaps with a slight preference for an AT-rich sequence around a strong transcriptional promoter. Consequently, pEPI-1 has no strong sequence preference for replication initiation in vivo (28). Therefore, we could not expect, and did indeed not look for, specific start sites in vitro. Instead, the purpose of the present study was to determine whether some of the known components of pre-RC are required for replication initiation in vitro. We describe here that nuclear extracts immunodepleted of Orc proteins or of Mcm proteins fail to induce plasmid replication in vitro.

The Orc proteins in nuclear extracts are organized in two major subcomplexes, Orc1–5 and Orc2–5, to which Orc6 may be loosely bound, if at all. Both subcomplexes can be removed from nuclear extracts by immunodepletion with Orc2-specific antibodies, whereas the Orc1–5 subcomplex is removed by immunodepletion with Orc1-specific antibodies. We have shown now that both depletion experiments resulted in a nuclear extract that failed to initiate replication in vitro. This indicates that the Orc1-containing subcomplex is essential, because the Orc2–5 subcomplex remains in the nuclear extract after treatment with Orc1-specific antibodies but is obviously unable to sustain replication in vitro.

We have tried to complement the depleted nuclear extract by a recombinant Orc1–5 subcomplex. This complex was stable in vitro and bound to DNA in an ATP-dependent manner like the recombinant ORC subcomplexes described by others (32, 43). In fact, it has been reported that an Orc1–5 subcomplex was fully active, complementing the replication activity of an immunodepleted Xenopus egg extract (4). However, the Orc1–5 subcomplex that we prepared failed to support DNA replication in the immunodepleted nuclear extract. The reason is not yet clear. One possibility is that antibody treatment removes an essential factor associated with the ORC or that the His tags on some of the recombinant Orc proteins or other modifications interfere with proper pre-RC formation in vitro.

We detected, however, that at least the dephosphorylated recombinant Orc1 subunit seems to function well in vitro, because it stimulates the overall replication activity when added to a nuclear extract. A plausible explanation is that recombinant Orc1 functions in place of endogenous Orc1, which dissociates from chromatin during S phase and is at least partially degraded in HeLa cells. In addition, we have shown that a partially purified ORC preparation from proliferating human cells is quite active complementing an immunodepleted nuclear extract. We conclude that Orc proteins are among the nuclear factors that promote plasmid replication in vitro.

We also conclude that Mcm proteins are essential for in vitro replication because removal of Mcm3 (with other associated Mcm proteins) inactivates the nuclear extract. This is interesting because the cytosolic S100 extract contains much Mcm3, which is, however, unable to substitute for the Mcm3 in the nuclear extract. In fact, not all Mcm proteins are loaded to chromatin, and substantial amounts are always found free in the nucleus, even at the end of the G1 phase when pre-RCs are poised for replication. The data shown here suggest that the fraction of unbound Mcms is not competent to initiate DNA replication in vitro. The function of the free Mcm proteins is not known. A possibility is that this fraction is marked for functions other than replication, e.g., for transcriptional regulation (44, 45).

The main intention of this report is methodological. It has shown that important components of pre-RC function in an in vitro system entirely composed of soluble proteins from proliferating mammalian cells. The major problem is that replication in vitro is slow and of low efficiency when compared with the well established T antigen-driven in vitro replication system. The most likely reason is that the various components of the pre-RC are present in low concentrations in nuclear extracts and interact at one of many sites on pEPI-1, whereas T antigen is offered in high concentrations, functions as a single protein, and targets a specific DNA site, the SV40 origin.

Thus, the replication efficiency of the cellular replication system must
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