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Purification of a Cellular Replication Factor, RF-C, That Is Required for Coordinated Synthesis of Leading and Lagging Strands during Simian Virus 40 DNA Replication In Vitro

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Cell extracts (S100) derived from human 293 cells were separated into five fractions by phosphocellulose chromatography and monitored for their ability to support simian virus 40 (SV40) DNA replication in vitro in the presence of purified SV40 T antigen. Three fractions, designated I, IIA, and IIC, were essential. Fraction IIC contained the known replication factors topoisomerases I and II, but in addition contained a novel replication factor called RF-C. The RF-C activity, assayed in the presence of I, IIA, and excess amounts of purified topoisomerases, was detected in both cytosol and nuclear fractions, but was more abundant in the latter fraction. RF-C was purified from the 293 cell nuclear fraction to near homogeneity by conventional column chromatography. The reconstituted reaction mix containing purified RF-C could replicate SV40 origin-containing plasmid DNA more efficiently than could the S100 extract, and the products were predominantly completely replicated, monomer molecules. Interestingly, in the absence of RF-C, early replicative intermediates accumulated and subsequent elongation was aberrant. Hybridization studies with strand-specific, single-stranded M13-SV40 DNAs showed that in the absence of RF-C, abnormal DNA synthesis occurred preferentially on the lagging strand, and leading-strand replication was inefficient. These products closely resembled those previously observed for SV40 DNA replication in vitro in the absence of proliferating-cell nuclear antigen. These results suggest that an elongation complex containing RF-C and proliferating-cell nuclear antigen is assembled after formation of the first nascent strands at the replication origin. Subsequent synthesis of leading and lagging strands at a eucaryotic DNA replication fork can be distinguished by different requirements for multiple replication components, but we suggest that even though the two polymerases function asymmetrically, they normally progress coordinately.

Eucaryotic chromosomes duplicate only during the S phase of the cell cycle by a highly controlled mechanism that allows only one round of DNA synthesis in each cycle. Previous studies with procaryotic systems have demonstrated that DNA replication is separated into multiple stages during which various replication machineries function (24, 25). In eucaryotes, little is known about the mechanism of DNA replication or the nature of the replication components, and thus more complex questions about control remain unanswered. For procaryotes and unicellular eucaryotes, such as yeast cells, one approach to resolving these questions has been the application of genetics, but it is almost impossible to apply simple genetics to mammalian cells. Fortunately, the development of a vigorous cell-free replication system for simian virus 40 (SV40) DNA (28, 29, 46, 50) has allowed, by direct biochemical means, the identification of cellular replication factors and elucidation of their functions. Indeed, a number of recent studies have characterized various cellular factors that are involved in SV40 DNA replication (20, 35, 39, 51, 53, 56).

SV40 is an ideal model system because it contains one origin of DNA replication and its chromosomal structure and mechanism of duplication closely resemble those of cellular chromosomes (15), where DNA replication is presumed to occur from multiple origins. Furthermore, replication is dependent on the cellular replication machinery, since only one virus-encoded protein, the SV40 large tumor antigen (TAg), is required. Efficient cell-free systems that support replication from the SV40 replication origin have been developed from permissive monkey cells (28) and semipermissive human 293 or HeLa cells (29, 46, 50). Replication in vitro requires the addition of purified TAg and a template DNA containing 65 base pairs (bp) of SV40 DNA, which constitutes the minimal core origin sequence. DNA synthesis initiates within the origin region, proceeds bidirectionally around the circular molecule, and terminates by a mechanism that resembles the process in cellular chromosomes. The final product is predominantly relaxed, covalently closed circular DNA, but with the addition of a nuclear extract, the system can assemble chromatin concomitant with DNA synthesis, with the consequent introduction of negative supercoils into the replicated molecules (44).

From analysis of both the kinetics of SV40 DNA replication in vitro and the deduced functions of proteins derived from the fractionated cellular extract, it has become clear that the replication cycle can be viewed as a series of distinct stages (22). Prior to initiation of DNA replication, multiple steps have thus far been recognized. The first is the ATP-dependent binding of SV40 TAg to its recognition sequences within the core origin (site II) and formation of a specialized nucleoprotein structure (6, 12–14). A second process, which may occur concomitantly with TAg binding, is the formation of an active "presynthesis complex" at the replication origin, and this step requires, in addition to TAg and template DNA, a cellular factor (20, 21, 47, 52, 54).

A third step, the unwinding of the duplex DNA at the origin, requires, in addition to the origin-binding function of TAg, a TAg DNA helicase activity. In the presence of ATP, TAg promotes local unwinding of the duplex SV40 origin sequence (7, 43). Extensive, bidirectional unwinding also

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requires a novel, multisubunit, single-stranded DNA-binding protein (referred to as HeLa SSB or RF-A), which has been identified in human cells as an essential factor for SV40 DNA replication (11, 17, 20, 51, 53, 54). Similar unwinding events prior to the initiation of DNA synthesis on duplex DNA templates occur in the *Escherichia coli oriC* (2) and bacteriophage λ (18) systems and are thus thought to be a common feature in the initiation events leading to duplex DNA replication.

In general, DNA replication forks proceed from the origin by semidiscontinuous DNA synthesis, in which one strand (the leading strand) at a replication fork is synthesized continuously while the lagging strand is synthesized discontinuously (36). For a long time it had been thought that the primary DNA polymerase for mammalian DNA replication was DNA polymerase α-primase complex. However, a second enzyme, DNA polymerase δ (10), has recently been implicated in replicative DNA synthesis, and an alternative viewpoint is that polymerases α and δ are responsible for lagging- and leading-strand synthesis, respectively (19, 38, 42, 45). This prediction was supported by the discovery that the proliferating cell nuclear antigen (PCNA; alternatively called cyclin) was required for SV40 DNA replication in vitro (39) and that this replication protein was an auxiliary protein that greatly stimulates the processivity of DNA polymerase δ (9, 19, 40, 48). Moreover, in the absence of PCNA, only lagging-strand DNA synthesis occurred during SV40 DNA replication in vitro (38). Although this is indirect evidence that polymerase δ is the leading-strand polymerase, it raised the possibility that two separate DNA polymerases function during elongation. At present, however, it remains an open question how these two DNA polymerases cooperate to synthesize duplex DNA at eucaryotic replication forks and, more specifically, how the two polymerases interact with each other following formation of the first nascent strands at the replication origin and what regulates this interaction.

In this report, we describe the purification of a novel replication factor, RF-C, that is required for elongation of DNA replication from the SV40 origin in vitro. As for PCNA, this factor is not required for initiation, but is required for subsequent synthesis of leading strands during the elongation stage of replication.

MATERIALS AND METHODS

[α-^{32}P]dATP (800 Ci/mmol) was obtained from Amersham. Restriction enzymes were purchased from New England BioLabs and used according to their instructions. Phosphocellulose (P-11) and hydroxylapatite were purchased from Whatman and Bio-Rad, respectively. Denatured DNA cellulose was prepared as described before (1). A Mono Q column (HR5/5) was obtained from Pharmacia and was run on the fast protein liquid chromatography (FPLC) system. Pancreatic DNase I-activated calf thymus DNA was prepared by a published procedure (3).

The plasmid DNAs used as templates (38) were pSVO10, which contains the entire SV40 genome in pUC18 and is 7.9 kilobases (kb) in size; pSVO11, which contains the SV40 origin fragment from *Hin*dIII (nucleotide [nt] 5171) to *Sph*I (nt 128) in pUC18 and is 2.9 kb in size; and pSVO2, which contains the entire SV40 genome in pAT153 and was used for the hybridization study, since this plasmid has no homology with the M13-SV40 probe DNAs except for the SV40 sequence. Single-stranded phage DNAs were prepared from six hybrid M13-SV40 clones described before (38).

SV40 TAg was obtained from HeLa cells coinfected with wild-type adenovirus and the recombinant Ad5SVR112 and was purified by immunoaffinity chromatography as described before (46).

Topoisomerases I and II were prepared from 355 g of calf thymus according to published procedures (30, 41) with slight modifications. Final preparations had specific activities of 2.4×10^7 U/mg for topoisomerase I and 4.5×10^5 U/mg for topoisomerase II. One unit was defined as the amount of enzyme that converted 50% of substrate DNA into relaxed forms in 25 μl of reaction mixture containing 0.5 μg of DNA at 37°C for 30 min.

Enzyme assays. DNA polymerase and topoisomerase activities in fractions were assayed as described before (10, 30, 41).

Replication reactions. Activity of SV40 DNA replication in vitro was assayed under standard conditions as described previously (46), with optimum amounts of purified SV40 TAg (1.0 to 1.5 μg), optimum amounts of individual fractions or the indicated combination of fractions, and 6 μg of template DNA per ml for 1 h at 37°C. Reactions were terminated with 10 mM disodium EDTA, and acid-insoluble counts were measured. Replication activities were expressed as picomoles of dAMP incorporated after incubation for 1 h. In the reconstituted reaction, RF-C activity was measured in the presence of purified TAg, 350 μg of fraction I*, 200 ng of purified topoisomerase I, and 90 ng of purified topoisomerase II in standard reaction conditions, and 1 U of RF-C activity represents the incorporation of 10 pmol of dAMP per h over the background obtained without RF-C (15 to 20 pmol).

Product analyses. To analyze the replication products, the reaction mixture containing the indicated amount of components was incubated at 37°C, and at the indicated time, the reaction was terminated by mixing with an equal volume of proteinase K solution (0.2 mg of proteinase K per ml, 2% sodium dodecyl sulfate [SDS], and 20 mM disodium EDTA), and the sample was incubated at 37°C for 2 h. DNA in the reaction mixture was extracted with phenol-chloroform (1:1), precipitated with ethanol, and dissolved in 20 μl of 10 mM Tris hydrochloride (pH 7.4)–1 mM disodium EDTA (TE). A portion of the sample was subjected to electrophoresis in a neutral agarose gel (0.8%) in TBE (32), in an alkaline agarose gel (1%) in 30 mM NaOH–1 mM disodium EDTA, or in a neutral agarose gel (1.5%) following digestion with restriction enzymes. Before the gels were dried for autoradiography, they were fixed in 10% methanol–10% acetic acid.

In hybridization studies with single-stranded M13-SV40 probe DNAs, product DNA from replication reactions was fragmented by digestion with *Rsa*I and *Dde*I, denatured at 100°C for 5 min, and hybridized with six M13-SV40 DNAs that had been blotted on a nitrocellulose filter as described previously (32, 38).

Preparation and fractionation of the cell extract for replication. Cytosol extracts and high-speed supernatants (S100) were prepared from 16 liters of suspended human 293 cells as described previously (28, 46). To obtain fractions I and II (Fig. 1A), the S100 fraction was adjusted to 0.2 M NaCl and loaded onto a phosphocellulose column (2.5 by 10 cm) that had been equilibrated in buffer A (25 mM Tris hydrochloride [pH 7.5], 1 mM disodium EDTA, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 0.01% Nonidet P-40 [NP-40], 1 mM dithiothreitol [DTT], 10% glycerol) containing 0.2 M NaCl, the column was washed with 100 ml of buffer A (0.2 M NaCl), and then bound proteins were eluted with 100 ml of buffer A (1 M NaCl). Proteins present in the unbound

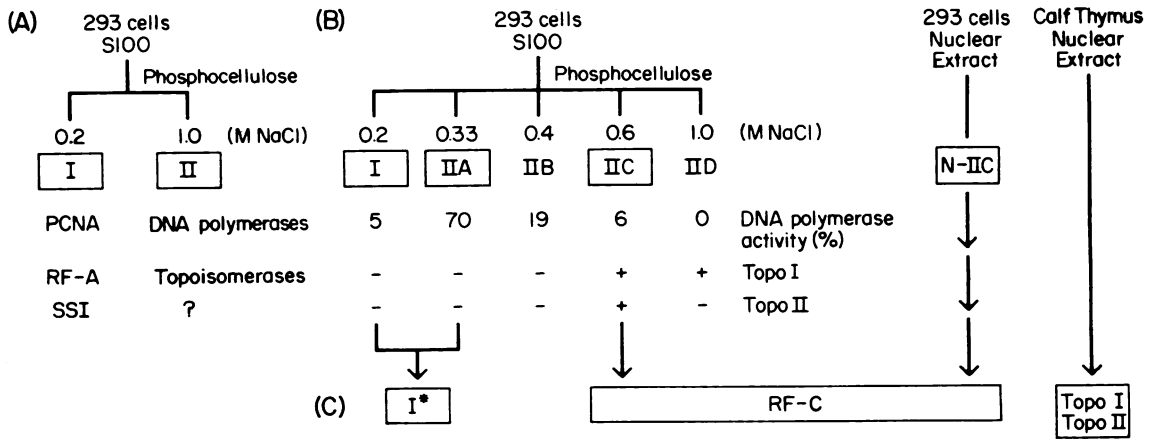

FIG. 1. Separation of cellular replication extracts into multiple components. (A) Fractionation scheme of the S100 extract from 293 cells for fractions I and II by phosphocellulose chromatography. Factors or fraction names that previously have been identified in these fractions are indicated. (B) Further fractionation of fraction II by phosphocellulose chromatography. Fractions essential for SV40 DNA replication are boxed. The DNA polymerase activity in each fraction is represented as a percentage of the total activity in each of the five fractions. Symbols in the topoisomerase (Topo) activity row indicate the presence (+) or absence (−) of topoisomerases I and II at more than 0.2 U/μl in each fraction. (C) The bottom line indicates components necessary for the reconstituted replication assay with RF-C. The source of each factor is presented. N-IIC, Fraction IIC obtained from the nuclear extract of 293 cells.

fraction (I) and the bound fraction (II) were identified by protein assay and then dialyzed against buffer A containing 0.025 M NaCl and 20% sucrose (39). To fractionate fraction II further (Fig. 1B), the bound proteins were eluted by successive washes with buffer A containing 0.33, 0.4, 0.6, and 1.0 M NaCl instead of a wash with buffer A containing 1.0 M NaCl. These four fractions were designated IIA, IIB, IIC, and IID, respectively, and were dialyzed as above. This fractionation scheme was modified again to prepare fraction I* (Fig. 1C), a combination of fractions I and IIA. In this case, the S100 was adjusted to 0.33 M NaCl and loaded onto a phosphocellulose column (2.5 by 10 cm) that had been equilibrated with buffer A plus 0.33 M NaCl, the column was washed with 100 ml of the same buffer, and thereafter, fractions IIB, IIC, and IID were eluted as above. Protein concentrations were determined by the method of Bradford (8) with bovine serum albumin as the standard.

Purification of RF-C. All of the procedures were done at 0 to 4°C. Nuclei derived from 16 liters of suspended 293 cells were obtained by centrifugation at 10,000 × *g* for 15 min after Dounce homogenization in hypotonic buffer (46). The nuclei were suspended in 50 ml of extraction buffer (25 mM Tris hydrochloride [pH 7.5], 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 0.15 M NaCl) and stirred for 30 min. This suspension was spun at 10,000 × *g* for 15 min, and the supernatant fraction, containing about 250 mg of protein, was obtained as the crude nuclear extract. In experiment 1 in Table 1, fraction IIC was prepared from a nuclear extract derived from 8 liters of suspended 293 cells. This fraction was loaded onto a hydroxylapatite column (1.2 by 4.5 cm) that had been equilibrated with buffer B (0.2 M potassium phosphate [pH 7.5], 1 mM DTT, 0.01% NP-40, 0.1 mM PMSF, 10% glycerol). The column was washed with 20 ml of the same buffer, and bound proteins were eluted with a 30-ml linear gradient from 0.2 to 0.5 M potassium phosphate in the same buffer. RF-C eluted in 0.25 M potassium phosphate, and fractions were pooled (4.4 ml), diluted with an equal volume of solution C (1 mM EDTA, 1 mM DTT, 0.01% NP-40, 0.1 mM PMSF, 10% glycerol), and loaded onto a phosphocellulose column (0.5 by 1.2 cm) equilibrated in buffer C (solution C containing 0.2 M potassium phosphate, pH 6.9). The column was washed with 1.5 ml of the same buffer, and proteins were eluted with a 6-ml linear gradient from 0.2 to 0.6 M potassium phosphate (pH 6.9) in buffer C. RF-C formed a broad peak, eluting in fractions centered at 0.3 M phosphate and a second sharper peak eluting at 0.48 M phosphate. Both of them contained the same RF-C proteins, as judged from their behavior in later chromatography steps; the difference seemed to be due to some modification of RF-C proteins or an association with other proteins. The 0.48 M peak had a higher specific activity than the 0.3 M peak and was purified further. The 0.48 M peak fractions were pooled (1.1 ml), diluted with 5.5 ml of solution C, and loaded onto a denatured DNA-cellulose column (1.2 by 0.5 cm) that previously had been equilibrated in buffer A plus 0.1 M NaCl. The column was subjected to four successive washes with 2.5 ml each of buffer A containing 0.1, 0.2, 0.33, and 0.66 M NaCl, respectively. Most of the RF-C activity eluted in the 0.66 M NaCl fraction, which was concentrated by binding to a small hydroxylapatite column and eluted by a wash with buffer B containing 0.3 M potassium phosphate. This fraction was used for replication product analysis.

For larger preparations of RF-C (experiment 2 in Table 1), every procedure (sizes of columns and amounts of buffers) from fraction IIC to the denatured DNA-cellulose step was scaled up by a ratio equivalent to the amount of starting material. Fractions containing RF-C were pooled from the denatured DNA-cellulose column, but the specific activity of this pool was lower than that of the equivalent fraction obtained in experiment 1. Therefore, this pool (1.6 ml, 5.9×10^3 U) was mixed with 15 ml of buffer D (same as buffer A except 25 mM Tris hydrochloride [pH 6.9]). This sample was loaded onto a Mono Q column that was equilibrated with buffer D containing 0.05 M NaCl. Proteins were eluted with 10 ml of a linear gradient from 0.05 to 0.6 M NaCl in buffer D, and RF-C eluted in fractions containing 0.2 M NaCl.

A portion (260 U of RF-C in 100 μl) of the peak fraction from the Mono Q column was loaded onto a 5-ml, 15 to 35% glycerol gradient in buffer A containing 0.1 M NaCl and subjected to centrifugation at 49,000 rpm in an SW50.1 rotor at 4°C for 24 h. The sample was fractionated into 41 fractions from top to bottom.

Protein analysis. Proteins were separated by SDS-polyacrylamide gel electrophoresis as previously described (26) and were visualized by silver staining by the method of Wray et al. (55) with minor modifications. Protein concentrations were determined by the method of Bradford (8).

RESULTS

Division of fraction II into multiple components. As described previously (39, 47), the cytoplasmic extract (S100) derived from human 293 cells was divided by phosphocellulose chromatography into two fractions, I and II, both of which were essential for SV40 DNA replication (Fig. 1A). From fraction I, the purified replication factors PCNA, RF-A (eucaryotic single-stranded DNA-binding protein), and a crude fraction SSI have already been identified as being essential for complete SV40 DNA replication in vitro (20, 39). Since preincubation at 37°C with TAg and fraction I eliminates a time lag for DNA synthesis, all of the replication factors in fraction II should work after the presynthesis stage. Indeed, replication factors such as DNA polymerases and topoisomerases I and II are present in this fraction (47). To analyze whether there were any unknown factors in fraction II, it was fractionated further by using the same resin (Fig. 1B). Since both polymerases α and δ were eluted from phosphocellulose with less than 0.35 M NaCl (27), proteins in fraction II were eluted initially with 0.33 M NaCl, and fraction IIA was obtained. As expected, this fraction contained most of the DNA polymerase activities from the S100 extract. Proteins remaining on the column were eluted into three fractions, IIB, IIC, and IID, with 0.4, 0.6, and 1.0 M NaCl, respectively.

Figure 2 shows the requirement for each fraction for SV40 DNA replication in a reconstituted reaction mix. In the presence of all of the fractions except fraction IIA, no DNA synthesis occurred, but upon addition of IIA, almost complete DNA synthesis occurred (Fig. 2A). However, only a small amount (5 to 10 pmol of dAMP) of DNA synthesis occurred in the presence of fractions I and IIA (Fig. 2B). This means that fraction IIA is essential, but other factors present in fraction IIB, IIC, or IID are also required for efficient replication. Addition of fraction IIC (without IIB and IID) enhanced incorporation to levels similar to those obtained with the crude S100 fraction (Fig. 2B). By reconstituting replication with all possible combinations of these fractions, it became apparent that fractions IIB and IID were not required for SV40 replication. It should be noted that the replicated product obtained with fractions I, IIA, and IIC was not identical to that obtained with the crude S100 extract (Fig. 3, lanes 1 and 2). In the reaction mix with these three components, high-molecular-weight products predominated, suggesting that the amount of topoisomerase in fraction IIC was not sufficient to completely segregate the replicated products, similar to previous observations by Yang et al. (56). Indeed, addition of purified topoisomerase I and topoisomerase II slightly stimulated the incorporation of label into replication products, but more significantly, the products were predominantly covalently closed, monomer molecules (Fig. 3, lane 5) (Tsurimoto and Stillman, manuscript in preparation). These results show that fraction II could be fractionated further into two essential fractions, IIA and IIC, by phosphocellulose column chromatography, and the combination of fractions I, IIA, and IIC was sufficient to yield replication products similar to those produced by the S100 extract. In the latter case, however, additional topoisomerase activity was required to produce fully replicated monomer molecules.

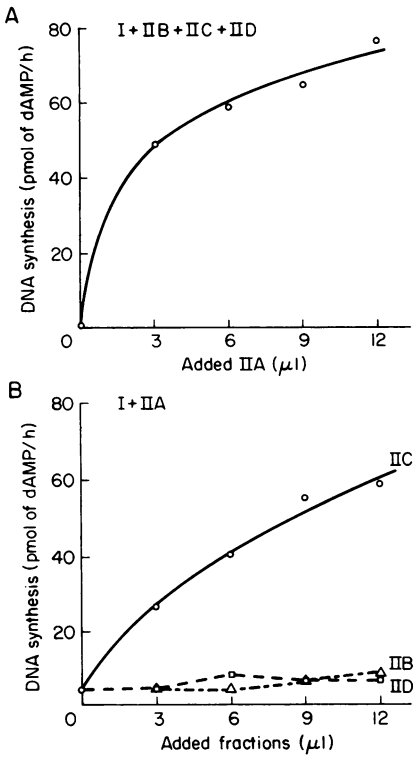

FIG. 2. Reconstitution of DNA replication with multiple fractions. (A) Titration of fraction IIA (3.4 mg/ml) in the presence of optimized, constant amounts of fraction I (230 μg) and a mixture of IIB, IIC, and IID (38 μg). (B) Titration of fractions IIB (0.9 mg/ml), IIC (1.7 mg/ml), and IID (1.2 mg/ml) separately in the presence of constant amounts of fractions I (230 μg) and IIA (34 μg).

Identification of RF-C activity in fraction IIC. Since fraction IIC contained limiting amounts of the known replication proteins topoisomerase I and topoisomerase II, we tested whether purified topoisomerases were able to substitute for fraction IIC. Purified topoisomerase I and topoisomerase II were added to reaction mixes containing fractions I and IIA in various amounts and in various ratios. Although purified topoisomerase I and topoisomerase II stimulated replication by as much as 30% of DNA synthesis levels obtained with the crude fraction IIC (Fig. 3, lanes 2, 3, and 4), the addition of topoisomerase I and topoisomerase II alone could not produce any completely replicated monomeric molecules. A small amount of form II molecules was observed, but they were produced by nonspecific DNA synthesis independent of the SV40 origin sequence and TAg and were sensitive to *Dpn*I digestion, which recognizes unreplicated molecules. Therefore, it is apparent that some unknown replication factor(s) other than topoisomerase I and topoisomerase II in fraction IIC is essential for SV40 DNA to replicate efficiently and completely. As expected, fraction IIC was not required for the presynthesis stage (data not shown); therefore, the factor(s) in fraction IIC was required for a subsequent stage. We called the activity present in this fraction RF-C (replication factor C) and started its purification, since it behaved as a single component in preliminary purification steps.

Reconstitution assay for RF-C. Replication in the presence of TAg and fractions I and IIA was limited (5 to 10 pmol of dAMP incorporated; Fig. 3, lane 3), and addition of the crude fraction IIC to the reaction mixes enhanced incorporation about 5- to 10-fold (Fig. 3, lane 2). This difference was

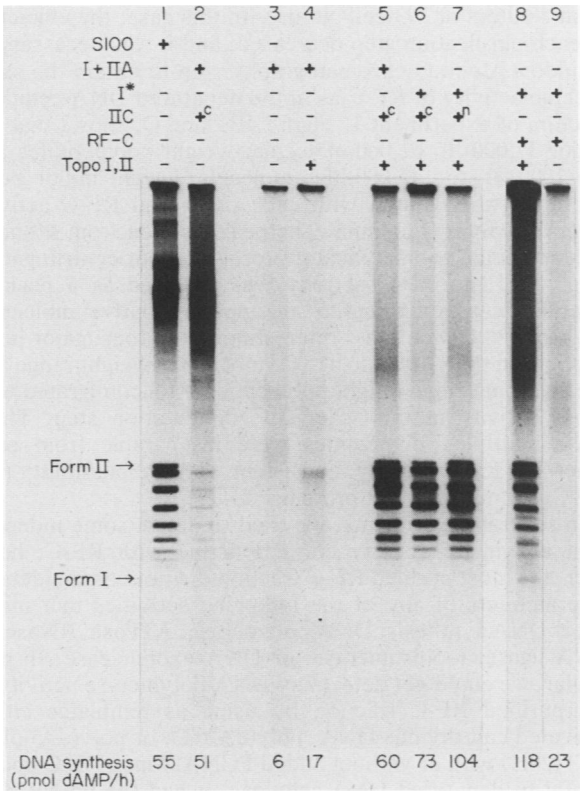

FIG. 3. Product analysis of SV40 DNA replication in the presence of various components. Used in these experiments were S100 (260 μg); fraction I (200 μg); fraction IIA (20 μg); cytoplasmic fraction IIC (+^{c}; 17 μg); nuclear fraction IIC (+^{n}: 7 μg); fraction I* (350 μg); topoisomerases I and II (200 and 90 ng, respectively); and RF-C (50 ng). Purified replication products from the reaction mixes were loaded onto a 0.8% agarose gel and subjected to electrophoresis at 2.5 V/cm for 10 h. After electrophoresis, the gel was fixed with 10% methanol–10% acetic acid, dried, and autoradiographed. The positions of forms I and II of the template plasmid (pSVO11) are indicated. Replication activity of each reaction mix is expressed by incorporation of dAMP in a 50-μl reaction mixture in 1 h and is shown below the lanes.

the result of the cooperative stimulation of DNA synthesis by RF-C, topoisomerase I, and topoisomerase II. Therefore, to assay RF-C during purification, it was important to use a reconstituted assay system that would be responsive only to added RF-C. Such a system could be obtained if, in combination with fractions I and IIA, an excess of purified topoisomerase I and II were used (see Materials and Methods). Although the amounts of added topoisomerases were about 10-fold in excess for topoisomerase I and 2-fold in excess for topoisomerase II over the amounts required for SV40 DNA replication (titration of these enzymes will be published elsewhere), they did not have any inhibitory effect on the replication reaction, and thus endogenous topoisomerases present in fraction IIC showed no additive incorporation. Under these conditions, the amount of incorporation with the five components (TAg, I, IIA, and topoisomerase I and II) was relatively high (15 to 20 pmol of dAMP per h), but addition of fraction IIC still exhibited approximately a threefold or greater stimulation of replication activity, which was due to RF-C (Fig. 3, lanes 4 and 5).

Since the ratio of the components in fractions I and IIA severely affected the amount of replication, this system was further simplified to keep a constant level of background replication in the absence of RF-C and a constant response to added RF-C. We therefore modified the phosphocellulose fractionation of the S100 extract to prepare fraction I* instead of the separated fractions I and IIA by loading the column in a buffer containing 0.33 M NaCl (Fig. 1C). Fraction I* had exactly the same properties as the combination of optimum amounts of fraction I and IIA, (Fig. 3, lane 6). Using fraction I* and excess amounts of topoisomerases I and II, we could quantitatively assay RF-C activity reproducibly.

Purification of RF-C. RF-C was originally identified in a subfraction derived from the 293 cell S100 extract, but it was also detected in a nuclear fraction (Fig. 1C). Proteins in the nuclei of 293 cells were extracted with various concentrations of NaCl, which yielded higher levels of RF-C (2×10^4 to 4×10^4 U from a 16-liter culture of 293 cells) in comparison with the amount present in the S100 extract (0.5×10^4 U from a 16-liter culture of 293 cells). RF-C was extracted from nuclei with 0.15 to 0.2 M NaCl, and extraction with higher salt concentrations did not increase the yield of RF-C. The proteins responsible for RF-C activity in the cytosol and nuclear extracts were identical, because both of them produced completely replicated products (Fig. 3, lanes 6 and 7), their chromatographic behavior was identical, and similar polypeptides were detected in the final stage of purification (data not shown). Therefore, we chose the 0.15 M NaCl nuclear extract as starting material for purification of RF-C.

Experiment 1 in Table 1 shows a representative purification of RF-C from 8 liters of 293 cell culture by phosphocellulose, hydroxylapatite, a second phosphocellulose, and denatured DNA-cellulose column chromatography. About 5 μg of nearly homogeneous RF-C was obtained following about 700-fold purification. SDS-polyacrylamide gel electrophoresis of the fractions from the denatured DNA cellulose column (Fig. 4) indicated that these fractions still contained

TABLE 1. Purification of RF-C from nuclear extract

Step no.	Fraction	Expt 1 (8-liter culture)					Expt 2 (48-liter culture)				
		Total protein (mg)	Total activity (U)	Sp act (U/mg)	% Recovery	Purification (fold)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	% Recovery	Purification (fold)
1	Nuclear extract	126	3.5×10^4	2.8×10^2	100	1	610	3.6×10^5	6.0×10^2	100	1
2	IIC	4.4	1.9×10^4	4.3×10^3	54	15	70	2.4×10^5	3.4×10^3	67	6
3	Hydroxylapatite	1.0	1.2×10^4	1.2×10^4	34	43	17	1.1×10^5	6.5×10^3	31	11
4	Phosphocellulose	0.22	4.1×10^3	1.9×10^4	12	68	4.5	4.0×10^4	9.0×10^3	11	15
5	Denatured DNA-cellulose	0.005	1.0×10^3	2×10^5	3	714	0.13	5.9×10^3	4.6×10^4	2	77
6	Mono Q						0.02	4×10^3	2×10^5	1	333

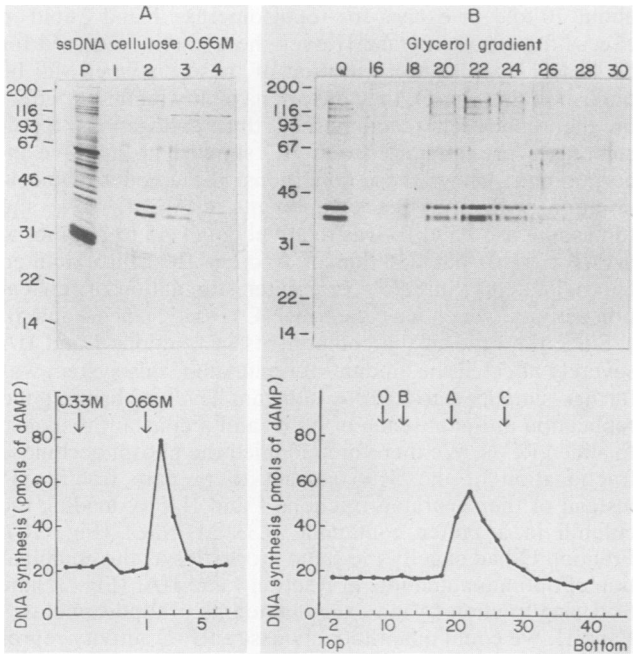

FIG. 4. Polypeptide composition of RF-C. (A) Elution of RF-C from a denatured DNA-cellulose column. (Upper panel) SDS-polyacrylamide gel (15%) of a portion of the indicated fractions eluted with 0.66 M NaCl. Lane P, Loading material for the column obtained from the second phosphocellulose pool. Positions of size markers (in kilodaltons) are indicated. (Lower panel) Elution of RF-C activity in the 0.66 M NaCl fractions. The number of each fraction corresponds to that of the lanes above. (B) Glycerol gradient sedimentation of RF-C. Centrifugation was done as described in the text. Every second fraction from the gradient was subjected to SDS-polyacrylamide gel electrophoresis (upper panel) and the SV40 DNA replication assay (lower panel). Numbers of the lanes correspond to the fraction number. Lane Q, Loading material for the glycerol gradient obtained from the Mono Q step. Protein markers sedimented in a parallel gradient were catalase (C, 250,000 daltons), alcohol dehydrogenase (A, 150,000 daltons), bovine serum albumin (B, 67,000 daltons), and ovalbumin (O, 45,000 daltons). Note that the background bands of 67,000 daltons which appear in some lanes are due to contamination.

several protein bands. Among those, polypeptides migrating with apparent molecular masses of 41,000 and 37,000 daltons were the major components, and the intensity of the bands exactly correlated with RF-C activity across the fractions. Interestingly, both of them appeared as doublet bands under different electrophoresis conditions (data not shown), and judging from their staining intensity, their molar ratio was roughly equal. The composition of these polypeptides was conserved in several independent experiments from different cell extracts and in further purification steps as described below. Thus, it is possible that RF-C is composed of a complex of these four polypeptides. It should be noted, however, that some minor bands appeared in the higher-molecular-mass region of the gel (100,000 to 140,000 daltons), and they also coeluted with RF-C activity.

To analyze the relationship between the major polypeptides and high-molecular-mass minor polypeptides that were found in the denatured DNA-cellulose fraction and to test the possibility that these major polypeptides were in a complex, we subjected this fraction to FPLC Mono Q chromatography and glycerol gradient centrifugation. Experiment 2 in Table 1 shows a larger preparation of RF-C from 48 liters of 293 cell culture. In this case, the efficiency of each purification step decreased, and it was necessary to include a Mono Q chromatography step to obtain the same specific activity of RF-C as in the denatured DNA-cellulose fraction of experiment 1. Figure 4B, lane Q, shows that the major 37,000- to 41,000-molecular-weight polypeptides and the 100,000- to 140,000-high-molecular-weight minor polypeptides still coeluted with each other and RF-C activity from the Mono Q column. The peak fraction from Mono Q was subjected to analytical glycerol gradient centrifugation (Fig. 4B), and RF-C activity was detected as a peak in fractions corresponding to an apparent native molecular mass of 180,000 daltons. Interestingly, the four major polypeptides in the 37,000- to 41,000-molecular-weight range and the high-molecular-weight polypeptides still comigrated with RF-C activity in this velocity sedimentation step. Thus, these multiple polypeptides were inseparable from each other and RF-C activity, consistent with the possibility that RF-C is a multisubunit protein.

In several experiments, we tried to detect some independent enzymatic activity cofractionating with RF-C; however, the most purified RF-C fraction did not contain detectable amounts of any of the following activities that might affect DNA synthesis: DNA polymerase, ATPase, RNase H, DNA ligase, topoisomerase, or DNA exonuclease. In particular, we could not detect any DNA polymerase activity in the purified RF-C fraction by using as templates either activated calf thymus DNA, poly(dA-dT), or poly(dA)-oligo (dT) (20:1) with or without added PCNA. Since RF-C bound tightly to denatured DNA-cellulose, it had the potential to bind some specific DNA substrates. However, we could detect neither specific binding to the SV40 core origin DNA nor preferential binding to denatured DNA, as has been found for other single-stranded DNA-binding proteins (20, 51, 53). Therefore, RF-C appears to bind DNA nonspecifically.

To test whether the purified RF-C could substitute completely for fraction IIC, the reaction product obtained with TAg, fraction I*, RF-C, and purified topoisomerases I and II was analyzed by agarose gel electrophoresis (Fig. 3, lane 8). These results clearly showed that SV40 DNA replicated efficiently and completely in this reconstituted reaction mix. If topoisomerases I and II were omitted from the reconstituted reaction mix containing purified RF-C, only limited DNA synthesis occurred and the product was not fully replicated (Fig. 3, lane 9). This result demonstrated that both RF-C and topoisomerases I and II are essential for complete replication. The role of each topoisomerase in this reconstituted reaction mix will be described elsewhere (Tsurimoto and Stillman, in preparation).

RF-C is required to replicate SV40 DNA completely. To study the function of RF-C further, the amounts of DNA synthesis and the structure of the replicated products were analyzed over the time course of SV40 DNA replication in a reconstituted system containing purified RF-C. We could more clearly demonstrate the effect of RF-C in the elongation stage of replication by using a large template plasmid (pSVO10 DNA, 7.9 kb) rather than the smaller plasmid (pSVO11, 2.9 kb) that was used in the experiments shown in Fig. 3. As shown in Fig. 5, SV40 DNA replicated more efficiently in the reconstituted system with saturating amounts of purified topoisomerases I and II, RF-C, and fraction I* than with the crude S100 extracts. The DNA synthesis rate with S100 slowed down after 60 min, but in the reconstituted system, DNA synthesis increased linearly for at least 90 min and incorporated over 200 pmol of dAMP. In

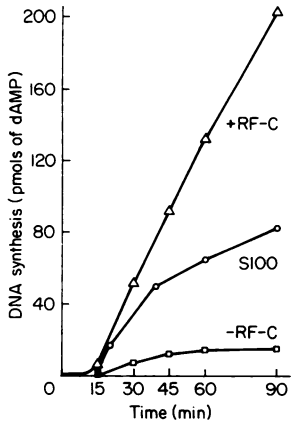

FIG. 5. Kinetics of SV40 DNA replication with the S100 extract or with the reconstituted system in the presence and absence of RF-C. Reaction mixtures with components similar to those described in the legend to Fig. 3 (i.e., fraction I* and topoisomerases I and II with and without RF-C, or S100 alone) were assembled on ice and incubated at 37°C for the indicated times. Samples were withdrawn at the indicated times and subjected to acid precipitation and product analysis (see Fig. 6).

other experiments, the DNA synthesis continued for more than 120 min and incorporated 280 pmol of dAMP, which corresponds to a 125% increase over the amount of input template DNA. As shown in Fig. 6A, the products of this reaction were almost the same as those obtained with the crude S100 system when analyzed by neutral agarose gel electrophoresis, suggesting that the high efficiency in the reconstituted reaction mix was due to the efficient production of completely replicated molecules and possibly to multiple rounds of DNA replication. Similarly, alkaline agarose gel electrophoresis (Fig. 6B) also demonstrated efficient elongation of DNA chains in the reaction mixes containing RF-C. At the 30-min time point, most of the DNA chains were elongated to full length and the rate of DNA synthesis was comparable with the DNA synthesis rate obtained with the crude S100 extract (data not shown). The electrophoresis pattern of restriction enzyme-digested product (Fig. 6C) also showed that with the completely reconstituted system, the DNA synthesis initiated at the SV40 origin region and elongated bidirectionally around the template, just as in the S100 system.

RF-C is required for coordinated synthesis of leading- and lagging-strand DNA. As described in the previous section, the replication reaction mixes without RF-C yielded a significant amount of DNA synthesis but fully replicated molecules were not produced (Fig. 3). When we carried out the reaction with the larger pSVO10 plasmid, the effect of RF-C on the reaction product was more pronounced (Fig. 5 and 6). The amount of DNA synthesis observed in reaction mixes containing RF-C compared with that in reaction mixes lacking this factor differed by more than 10-fold at the later time points (Fig. 5). Thus, the structure of the replication products from reaction mixes which lacked RF-C was investigated and compared with those of the fully reconstituted system.

In the following experiments, it should be noted that the total amount of label incorporated into replication products was much greater in the presence of RF-C than in its absence (see Fig. 5); however, to compare the products on gels, an equal number of counts were generally compared. Figure 6A demonstrates that at early time points in the replication reaction without RF-C (15 to 45 min), a labeled species that migrated in neutral agarose gels slightly more slowly than form I marker DNA was observed, with a minor form migrating more slowly than form II marker DNA. These topologically related species are similar to the early replication intermediates previously described by Prelich and Stillman (38). They disappeared upon prolonged incubation, and a smear of high-molecular-weight products was observed. In addition, a significant amount of small DNA was found migrating in the 100- to 300-bp range, again reminiscent of the replication products observed in the absence of PCNA (38). When these replication products were denatured and subjected to alkaline gel electrophoresis, the majority of the DNA was approximately 100 to 300 nt long (Fig. 6B). Finally, when the products were digested with restriction enzymes and subjected to agarose gel electrophoresis, the majority of the label migrated in the low-molecular-weight range, whereas the template-bound duplex DNA derived predominantly from the origin region (Fig. 6C). Again, these analyses suggested that the replication products synthesized without RF-C were the result of an elongation block and were similar in nature to the products observed without the elongation factor PCNA (38).

In the absence of PCNA, replication intermediates that contained short nascent strands at the replication origin and migrated slightly more slowly than forms I and II accumulated in the replication system. Interestingly, when elongation continued from these early replication intermediates, most of the nascent DNA synthesized in the absence of PCNA was displaced from the template, was approximately 200 bases in length, and hybridized to the noncoding, single-stranded probes derived from the SV40 genome. It was concluded that in the absence of PCNA, only lagging-strand synthesis was occurring following the synthesis of the first nascent strands at the replication origin; however, the lagging-strand products were not ligated and were displaced from the template DNA. Since omission of RF-C from the reconstituted reaction mix produced almost the same replication intermediates and short DNA strands, it was important to analyze their strand specificity in an analogous manner.

Replication reactions were carried out with and without RF-C for 40 min, and the products were isolated and hybridized to single-stranded M13-SV40 DNA that had been blotted onto nitrocellulose filters. As indicated in Fig. 7, in the presence of RF-C, the products hybridized equally with both leading- and lagging-strand-specific probe DNAs. Therefore, the reconstituted reaction mix containing RF-C was capable of synthesis of both strands with the same efficiency. When RF-C was omitted from the reaction mix, the products hybridized predominantly with the lagging-strand-specific DNAs (noncoding DNAs) from both sides of the origin. The results of these hybridization studies are similar to those obtained with and without PCNA and suggested that in the absence of RF-C, only lagging-strand synthesis occurred after the initiation of replication of the origin, but the lagging-strand product was abnormal.

As shown previously (Fig. 3), topoisomerases I and II were also essential for complete replication. Therefore, comparable hybridization experiments were done in the absence of topoisomerases I and II or in the absence of both RF-C and topoisomerases I and II. In both cases, the incorporation of dAMP was very limited and early replication intermediates accumulated (data not shown). However, when we applied the products from these experiments to the

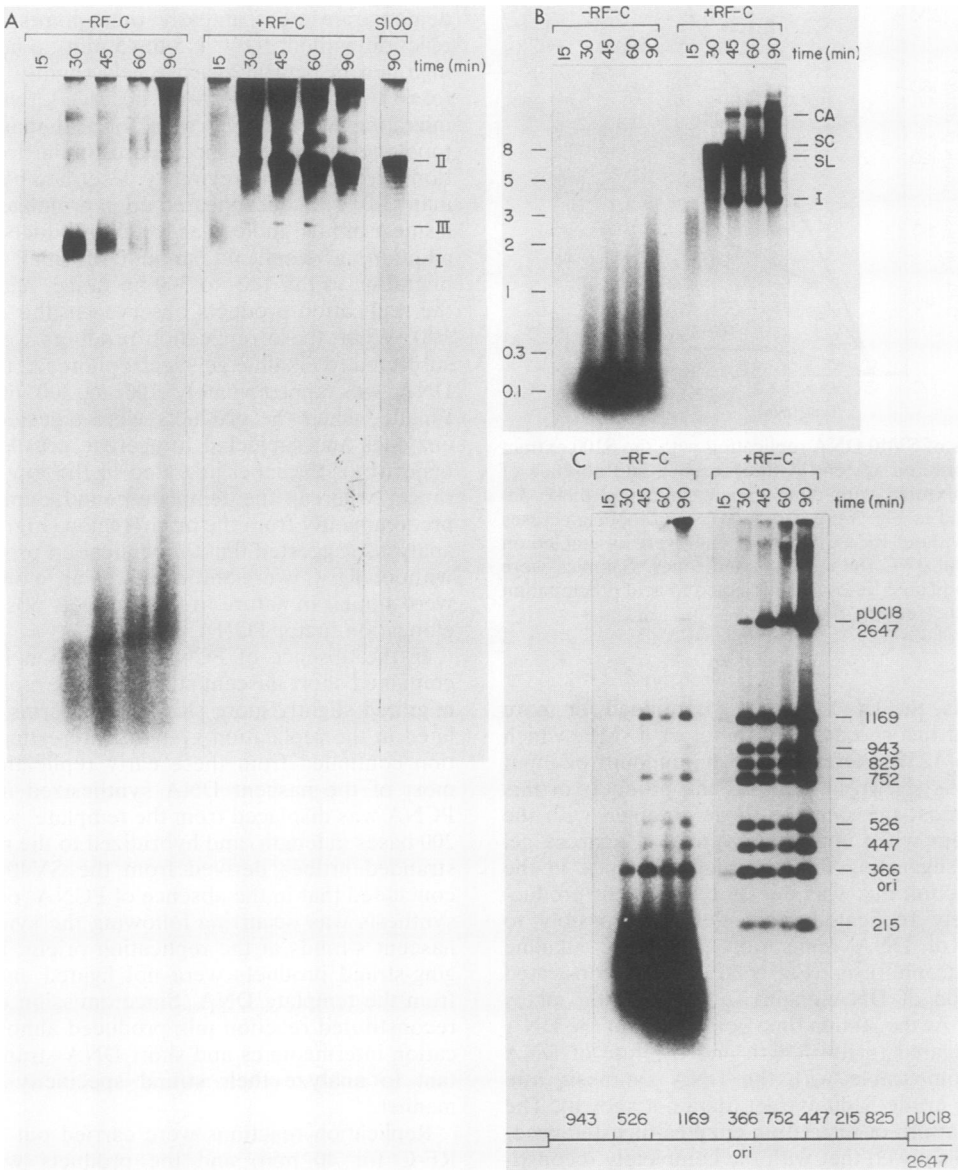

FIG. 6. Analysis of the replication products synthesized in the presence and absence of RF-C. All reaction mixes contained optimum amounts of TAg, fraction I*, and topoisomerases I and II in the presence or absence of RF-C as shown and were incubated at 37°C for the times indicated. Replicated products from the indicated time points were purified, and samples containing approximately the same number of counts were loaded directly into a neutral agarose gel (A) or, following denaturation, into an alkaline agarose gel (B), except for the 15-min samples, in which the counts were about one fifth of those in the other samples. (A) Electrophoresis conditions were the same as in Fig. 3, and mobilities of forms I, II, and III of the template DNA (pSVO10) are indicated. The product of a reaction mix containing S100 extract at 90 min is shown as a control. (B) The alkaline agarose gel was run at 1.5 V/cm for 12 h. Mobilities of denatured DNA fragments were obtained from *Hin*dIII-digested adenovirus type 2 DNA and are shown in kilobases on the left. Other markers designated I, SL, SC, and CA are denatured form I, single-stranded linear, single-stranded circular, and denatured catenated molecules of unit length of template DNA, respectively. (C) Replication products from the time points (in this case, an equal portion of each sample was subjected to the analysis) were digested with *Bam*HI, *Hin*dIII, and *Kpn*I and subjected to electrophoresis in a 1.5% agarose gel at 2 V/cm for 10 h. The size (in base pairs) of each digested band is indicated on the right, and the corresponding map is shown below the autoradiograph. Fragments containing the SV40 replication origin and flanking SV40 DNA (thin line) and vector plasmid pUC18 DNA (boxed) are shown.

same hybridization protocol, different results were obtained. Unlike reaction mixes deficient in RF-C, products from reaction mixes without topoisomerase hybridized equally with both strand-specific probe DNAs (Fig. 7). When both topoisomerases and RF-C were omitted, the hybridization pattern resembling that of the reaction mix without RF-C again appeared. In summary, RF-C appears to be required for coordinated synthesis of leading and lagging strands, and this is independent of the presence of topoisomerases I and II. Interestingly, the products from the reaction mixes lacking topoisomerase hybridized more to the SV40 origin-specific probe DNAs (ori1 and ori2) than those from reaction mixes which contained topoisomerases. Therefore, topoisomerases I and II are not required for coordinated synthesis

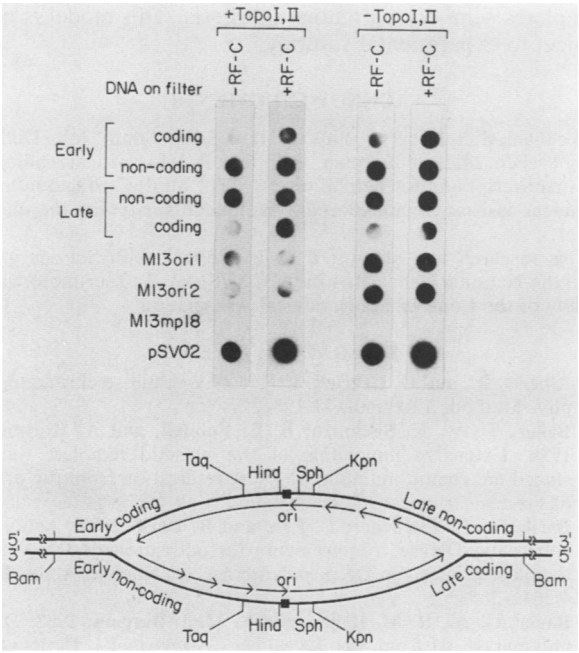

FIG. 7. Strand specificity of replication in the reconstituted reaction mix in the presence and absence of RF-C and topoisomerases I and II (Top) Reaction mixes containing plasmid pSVO2, TAg, fraction I*, and the indicated components were incubated for 40 min at 37°C, and the strand specificity of the synthesized DNA was analyzed by hybridization to 0.1 μg of single-stranded M13-SV40 probe DNAs which had been immobilized onto nitrocellulose filters, as indicated on the left. The late-region coding and noncoding strands were from the *Kpn*I-*Bam*HI fragment, and the early coding and noncoding strands were from the *Taq*I-*Bam*HI fragment. The *ori* strands were from the *Hind*-*Sph* fragment. pSVO2 was denatured and contained the entire template sequence. (Bottom) Schematic diagram of a replication intermediate, showing the location of the single-stranded regions used for the experiment and the leading (long arrows) and lagging (short arrows) strands on each side of the origin (solid squares).

of leading and lagging strands, but are required for replication fork progression around the template DNAs (56; Tsurimoto and Stillman, in preparation).

DISCUSSION

In this study, components from a 293 cell cytosol extract (S100) that are required for SV40 replication were fractionated into several distinct classes by phosphocellulose chromatography. Initially the extract was divided into five fractions, and three of them, fractions I, IIA, and IIC, were found to be essential. Further purification revealed three necessary and sufficient components in fraction IIC: topoisomerases I and II and a novel replication factor called RF-C.

Approximately three times more RF-C activity was present in the nuclear extract than in the cytoplasmic extract. This was not due to the presence of a different stimulatory factor in the nuclear extract, since the specific activity of purified RF-C from both sources was the same (data not shown). The tight association of RF-C with the nucleus is different from that of some other replication factors that have been identified as essential for SV40 DNA replication. Polymerase α, PCNA, and RF-A, a eucaryotic single-stranded DNA-binding protein, were quantitatively extracted from the nucleus into the cytosol extract with the low-salt hypotonic buffer, although they were present in nuclei in intact cells (data not shown) (5). Since the RF-C activity was efficiently eluted from nuclei with a buffer containing 0.15 M NaCl, it has an intermediate nuclear affinity that might represent an interaction of RF-C with chromosomal DNA or other nuclear components.

Since fraction I* and excess amounts of topoisomerases I and II could provide all of the replication functions other than RF-C, its activity could be measured even in crude fractions. By using reconstitution of replication as an assay, RF-C was purified as a single component by four or five chromatographic steps. However, the most purified form of RF-C (from glycerol gradient fractions in experiment 2, Table 1, and Fig. 4B) still contained several polypeptides. The major protein components that coeluted with RF-C activity from denatured DNA-cellulose and Mono Q columns and during glycerol gradient sedimentation were two protein doublets with apparent molecular masses of 37,000 and 41,000 daltons. Their stoichiometry remained roughly 1:1:1:1 from preparation to preparation. The native molecular mass of RF-C was approximately 180,000 daltons from the glycerol gradient experiment, suggesting that a complex containing these four polypeptides was responsible for the RF-C activity. However, since four high-molecular-weight polypeptides ranging from 100,000 to 140,000 were also closely associated with RF-C activity in these steps, we cannot rule out the possibilities either that the high-molecular-weight polypeptides are true components of RF-C or that both the high- and low-molecular-weight species combine to constitute RF-C activity. This problem may be resolved by isolation of monoclonal antibodies for each of these polypeptides, and this work is ongoing at present.

RF-C is not required for formation of the presynthesis complex, the subsequent unwinding reaction, or formation of the first nascent strands at the replication origin, but in the absence of RF-C, the amount of DNA synthesis is greatly reduced and all of the products of this reaction are replication intermediates. Therefore, RF-C is required for correct elongation of DNA synthesis. Additional factors such as polymerase α, PCNA, and topoisomerases I and II have been identified as elongation factors (35, 39, 56), but RF-C can be physically separated from them by column chromatography. Since we could not detect any independent enzymatic activities for RF-C, it is probably distinguishable from other known or suspected replication activities, such as DNA ligase, exonucleases, RNase H, and DNA helicase, that could affect the elongation of DNA synthesis (16, 23, 37). Vishwanatha et al. also reported two factors that stimulated polymerase α activity (49), but their chromatographic behavior and molecular weights were different from those of RF-C. Therefore, RF-C is a novel elongation factor that is essential for SV40 DNA replication and probably has a novel function in DNA replication.

Replication reaction mixes that lacked RF-C yielded products similar to those of reaction mixes that lacked PCNA. Omission of either of these replication proteins from the reconstituted reaction mix resulted in the accumulation of species that resembled early replication intermediates and displaced nascent short DNA strands of about 200 bases in length that were not ligated. Most of the short nascent DNA strands hybridized to the lagging-strand templates, demonstrating that abnormal lagging-strand synthesis proceeded in the absence of PCNA or RF-C, but in addition, leading-strand replication did not occur. In the absence of either of these replication proteins, initiation at the replication origin appeared to occur normally, but there was a block to the

formation of a functional elongation complex. Thus, both RF-C and PCNA are required to switch DNA synthesis from the initiation mode to the elongation mode, but we cannot yet distinguish which protein is required first.

Since double-stranded DNA genomes contain antiparallel, complementary DNA strands, replication occurs by a semi-discontinuous mechanism, in which synthesis of leading strands at the replication fork proceeds continuously and synthesis of lagging strands proceeds discontinuously. Therefore, replication at a fork occurs asymmetrically. Mammalian cells have two potential replicative DNA polymerases, polymerase α and possibly polymerase δ, that might function to replicate lagging and leading strands, respectively (19, 38, 42, 45). PCNA was identified as an auxiliary protein for polymerase δ that switches it from a nonprocessive to a processive polymerase (40, 48). Thus, the requirement for PCNA for leading-strand replication could easily be explained if polymerase δ were the leading-strand polymerase (38); however, the absence of PCNA also affects lagging-strand synthesis, suggesting that normal replication of both strands occurs coordinately. Recently, it was proposed that a functionally heterogeneous dimer of *E. coli* DNA polymerase III holoenzyme works at a replication fork in *E. coli* cells (31, 34). In addition, yeast DNA polymerase III, an enzyme analogous to polymerase δ, has been identified in *Saccharomyces cerevisiae* (4). Therefore, it might be a general feature of DNA replication that functionally heterogeneous DNA polymerases and/or polymerase subunits synthesize leading and lagging strands but combine to form a replisome polymerase unit at a replication fork.

Although we have not determined the precise function of RF-C in DNA replication, several possible functions come to mind. First of all, because we have been unable to detect any DNA polymerase activity with several template DNAs and in the presence or absence of PCNA, we consider it unlikely that RF-C is a form of polymerase δ. One function could be that RF-C is another auxiliary protein for polymerase δ that stimulates its processivity, similar to PCNA. But polymerase δ is very processive in the presence of PCNA alone, and the presence of additional subunits such as RF-C has not been reported. A second possibility is that RF-C pushes the replication fork along by destabilizing the duplex DNA in much the same way as a DNA helicase or unwinding DNA-protein complex acts in front of the DNA polymerases at the replication fork. If this were so, there would have to be an additional mechanism involving RF-C to explain the strand-specific replication by the DNA polymerases. We think this is unlikely and also note that topoisomerases I and II can function as a swivelase for DNA replication, yet there is no strand specificity for DNA replication in their absence. A third possibility is that RF-C may facilitate coordinated synthesis of both the leading and lagging strands, perhaps acting as a connector or hinge between the two polymerase complexes, in much the same way as the tau subunit of the *E. coli* polymerase III subunit may function (33).

In conclusion, it is clear that initiation of DNA replication can occur in the absence of RF-C and PCNA, and our preliminary evidence suggests that polymerase α-primase complex is responsible for synthesis of the first nascent strands at the replication origin. We propose that this polymerase might then move away from the origin with the replication fork and continue to synthesize the lagging strand. At a subsequent stage that involves both RF-C and PCNA, the leading-strand polymerase complex is then assembled onto the other strand (leading-strand template), ultimately to form a coordinated replication complex that completes semi-discontinuous synthesis. This model is now subject to experimental scrutiny.

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