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Reconstitution of simian virus 40 DNA replication with purified proteins

(DNA polymerase δ/replication factor C/eukaryotic DNA replication)

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ABSTRACT **Replication of plasmid DNA molecules containing the simian virus 40 (SV40) origin of DNA replication has been reconstituted with seven highly purified cellular proteins plus the SV40 large tumor (T) antigen. Initiation of DNA synthesis is absolutely dependent upon T antigen, replication protein A, and the DNA polymerase α–primase complex and is stimulated by the catalytic subunit of protein phosphatase 2A. Efficient elongation of nascent chains additionally requires proliferating cell nuclear antigen, replication factor C, DNA topoisomerase I, and DNA polymerase δ. Electron microscopic studies indicate that DNA replication begins at the viral origin and proceeds via intermediates containing two forks that move in opposite directions. These findings indicate that the reconstituted replication reaction has many of the characteristics expected of authentic viral DNA replication.**

The replication of chromosomal DNA in mammalian cells is not yet understood. To gain better insight into this process, we have been studying the replication of the genome of the papovavirus simian virus 40 (SV40) (1, 2). SV40 DNA replication takes place in the nucleus of permissive primate cells. Except for the participation of a single virus-encoded protein, the SV40 large tumor (T) antigen, viral DNA replication is dependent upon the resident cellular replication machinery. For this reason the fundamental mechanisms of SV40 DNA replication are probably quite similar to those of chromosomal DNA replication. Thus, the detailed biochemical analysis of SV40 DNA replication represents an attractive approach to the identification and functional characterization of cellular DNA replication proteins.

We previously established a crude *in vitro* system capable of supporting the complete replication of plasmid DNA molecules that contain the SV40 origin of DNA replication (3). This system consists of a soluble extract from permissive cells supplemented with purified SV40 T antigen. DNA replication in the system exhibits most of the characteristics of SV40 DNA replication *in vivo* (3–7). In particular, DNA synthesis begins in the neighborhood of the SV40 origin and proceeds bidirectionally via replication intermediates identical to those observed *in vivo*. We and others have made use of the cell-free SV40 DNA replication system to explore the enzymatic mechanisms of viral DNA replication and to identify the proteins involved (1, 2). These studies have provided evidence for the involvement of a number of cellular proteins in SV40 DNA replication *in vitro*: DNA polymerase α–primase complex (8, 9), topoisomerases I and II (10), replication protein A (RP-A) (11–13), proliferating cell nuclear antigen (PCNA) (14), the catalytic subunit of protein phosphatase 2A ($PP2A_c$) (15, 16), replication factor C (RF-C) (17), and DNA polymerase δ (18–20).

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A complete understanding of the biochemical mechanisms involved in SV40 DNA replication will require the development and analysis of a purified protein system capable of supporting the complete replication reaction *in vitro*. In a previous paper we described a general approach to the fractionation of the crude SV40 DNA replication system and demonstrated that efficient DNA synthesis could be reconstituted with five highly purified and two partially purified cellular fractions (9). Further fractionation of the crude system has resulted in the resolution of the partially purified fractions, and we now report the reconstitution of SV40 DNA replication with seven highly purified cellular proteins plus the SV40 T antigen. The reconstituted replication reaction has many of the characteristics expected of authentic viral DNA replication and should provide the basis for more detailed mechanistic studies.

MATERIALS AND METHODS

Reagents and Enzymes. Reagents were obtained from the sources indicated previously (9, 19) with the following exceptions: topoisomerase I, Bethesda Research Laboratories; Mono S chromatography matrix, Pharmacia; and single-stranded DNA cellulose, Sigma. Preparation of HeLa crude cytoplasmic extract; cellular fractions CF I, CF II, CF IIA; and the purified proteins PCNA, RP-A, $PP2A_c$, DNA polymerase α–primase complex, and T antigen have been described (9, 12, 15, 16, 19).

Purification of CF IIB (RF-C). CF IIB was identified as a component of CF II (9) required for reconstitution of SV40 DNA replication. CF II was separated into two fractions, CF IIB and CF II′, by phosphocellulose chromatography. Fraction CF II′ was eluted at 0.4 M KCl in buffer H (9), and fraction CF IIB was eluted at 1 M KCl. Both fractions were required for efficient SV40 DNA replication *in vitro*. CF IIB activity was subsequently assayed by complementation in standard (9) 25-μl reaction mixtures containing 19 μg of CF I, 8.7 μg of CF II′, 1 μg of T antigen, and 50 ng of pUC.HSO DNA. Since chromatographic properties of CF IIB were found to be similar to those of RF-C (17), large scale purification of CF IIB (RF-C) was carried out by using a modified version of the protocol described by Tsurimoto and Stillman (17). Nuclei from 48 liters of S3 HeLa cells were prepared as described (21). CF IIB activity was fractionated as described (17), except that a concentration step on a 3-ml hydroxylapaptite column was substituted for the second

Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen; RP-A, replication protein A; PCNA, proliferating cell nuclear antigen; $PP2A_c$, catalytic subunit of protein phosphatase 2A; RF-C, replication factor C; CF, cellular fraction.

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phosphocellulose column. The final yield of CF IIB (RF-C) was approximately 70 μg, which represented 10% of the starting activity.

DNA Polymerase δ. DNA polymerase δ was purified from CF II, which had been depleted of polymerase α by SJK237 immunoaffinity chromatography (9). Depleted CF II (CF IIA) was fractionated on a 10-ml Mono Q column and assayed as described (19). The active fractions (2500 polymerase units; 8 mg of protein) were diluted 1:1 and loaded onto a 1-ml Mono S column equilibrated in buffer A (19) containing 25 mM KCl. The column was eluted in 5-ml steps of 25 mM, 300 mM, 400 mM, 500 mM, and 1 M KCl in buffer A. Polymerase δ activity eluted at 400 mM KCl. Thirty percent of the polymerase activity was recovered with a 6-fold purification. A fraction of the Mono S peak was diluted 1:1.5 in buffer B (30 mM Hepes, pH 7.8/0.25 mM EDTA/0.25% inositol/25 mM KCl/1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride) containing 15% glycerol and loaded onto a 5-ml glycerol gradient (15–40% glycerol in buffer B). The gradient was centrifuged in an SW 50.1 rotor at 44,000 rpm for 37 hr. About 50% of the polymerase activity was recovered. Protein levels in the peak fractions were below the limit of detection; however, estimates of protein recovery from silver-stained gels indicated >100-fold purification.

DNA Replication Assays. SV40 replication reactions with purified proteins were performed under the conditions described by Wold *et al.* (9). Standard 25-μl reaction mixtures contained 80 ng of SV40 origin-containing plasmid pUC.HSO (22) or pSV5 (4), 350 ng of T antigen, 420 ng of RP-A, 100 ng of $PP2A_c$, 10 units of topoisomerase I, 0.12 unit of polymerase α–primase complex, 75 ng of RF-C, 30 ng of PCNA, 0.1–0.2 unit of polymerase δ, and 10 μCi (1 Ci = 37 GBq) of [α-^{32}P]dCTP. Denatured samples were heated to 100°C for 5–10 min prior to electrophoresis on a 1% agarose gel. Hybridization of the products to strand-specific probes on nitrocellulose filters was performed as described (23). The probes were prepared from six phage M13–SV40 hybrid clones (gift of M. Depamphilis, Roche Institute of Molecular Biology) containing the individual strands derived from the following three segments of SV40: nucleotides 5092–160 (clones MSV01 and MSV02), 294–551 (clones MSV09 and MSV10), and 1620–1988 (clones MSV07 and MSV08).

Electron Microscopy. Samples for microscopy were isolated from reconstituted replication reactions in which pSV5 DNA linearized with *Sca* I served as template (4). After 50 min of incubation, the reactions were terminated with proteinase K, sodium dodecyl sulfate, and EDTA (9). The samples were extracted with 1:1 (vol/vol) phenol/chloroform and dialyzed against 10 mM Tris·HCl, pH 8.0/1 mM EDTA. Microscopy and length measurements were performed as described (4, 24).

RESULTS

Cellular Proteins Required for SV40 DNA Replication. To identify and characterize the cellular proteins required for SV40 DNA replication, we initiated a program of systematic fractionation–reconstitution experiments (9). A total of seven cellular replication proteins required for efficient viral DNA replication have now been resolved from the crude extract and purified to near homogeneity. These include RP-A (RF-A), DNA polymerase α, $PP2A_c$, PCNA, RF-C, topoisomerase I or II, and DNA polymerase δ. We previously have described the purification of five of the seven required cellular proteins (9, 10, 12, 15, 16, 25). The purification of human DNA polymerase δ and RF-C is described in *Materials and Methods*.

The biochemical properties of the purified human DNA polymerase δ were similar to those reported for calf thymus DNA polymerase δ (26) and the partially purified human enzyme (19). In particular, DNA polymerase activity was almost completely dependent upon the accessory protein PCNA when poly(dA)·oligo(dT) was used as template primer. The purified enzyme contained no detectable primase activity but did exhibit a low level of 3′-to-5′ exonuclease activity specific for single-stranded DNA. Further work will be required to determine whether this 3′ exonuclease activity is intrinsic to the enzyme. DNA polymerase δ activity cosedimented with DNA replication activity detected by the reconstitution assay, indicating that the enzyme is required for efficient SV40 DNA replication *in vitro* (data not shown). The most highly purified fraction contained a major polypeptide of 166 kDa and less prominent polypeptides of 210, 66, 54, and 46 kDa that cosedimented with DNA polymerase activity in the final glycerol gradient step of the purification. We are not certain at this point whether all of these polypeptides are physically associated.

An additional component of the cytoplasmic extract required for reconstitution of SV40 DNA replication, which we initially designated CF IIB, was tentatively identified as RF-C (17) on the basis of its chromatographic properties during purification. The identification of CF IIB as RF-C was confirmed by analysis of the physical and biochemical properties of the purified protein. Like RF-C (17), the purified CF IIB protein contained three major polypeptides of 38, 39, and 40 kDa and a number of minor polypeptides with molecular masses greater than 100 kDa. In addition, the protein exhibited DNA-dependent ATPase activity. When the protein was incubated with [α-^{32}P]ATP and irradiated with UV, the 40-kDa polypeptide was labeled with ^{32}P, indicating that it has a binding site for ATP (data not shown).

Properties of the Reconstituted DNA Replication Reaction. Fig. 1 shows the time course of the SV40 DNA replication reaction reconstituted with T antigen and the seven purified cellular proteins, RP-A, PCNA, DNA polymerase α–primase complex, topoisomerase I, $PP2A_c$, RF-C, and DNA polymerase δ. Like DNA replication in crude extracts, the reconstituted reaction exhibited a lag of 15–30 min after which incorporation of precursor into DNA was linear for about 1 hr. The total quantity of DNA synthesized in a standard reaction (5–40 pmol) was generally less than that observed with crude extracts, but under optimal conditions the newly synthesized DNA amounted to ≈50% of the input DNA.

We also observed significant DNA synthesis in reaction mixtures containing only T antigen, RP-A, $PP2A_c$, DNA polymerase α–primase complex, and topoisomerase I (Fig. 1). Thus, this subset of proteins appears to be sufficient for initiation of DNA synthesis on duplex DNA molecules containing the SV40 origin of replication. The time course of this

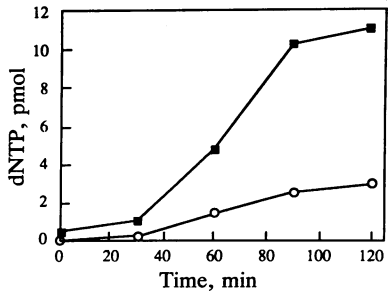

FIG. 1. Time course of DNA synthesis in the reconstituted system. Complete reconstitution reactions (■) contained purified T antigen, RP-A, $PP2A_c$, topoisomerase I, DNA polymerase α–primase complex, DNA polymerase δ, RF-C, PCNA, and pSV5 as described. Partial reconstitution reactions (○) lacked PCNA, RF-C, and DNA polymerase δ. Reaction mixtures were incubated at 37°C, and samples were removed at the indicated times for assay of radioactivity incorporated into replication products.

partial reaction was similar to that of the complete reaction, but the total amount of DNA synthesized was significantly reduced, and the newly synthesized strands were shorter (Fig. 1 and see below). The partial reaction also differed from the complete reaction in the strand specificity of DNA synthesis (Table 1). Partial and complete replication reactions were carried out with pSV5 DNA, which contains the complete SV40 genome. The products of each reaction were hybridized to strand-specific M13–SV40 probes corresponding to different segments of the viral genome. The DNA synthesized in the reaction lacking DNA polymerase δ, PCNA, and RF-C hybridized preferentially to the lagging strand template (except for the products that originated from the segment containing the origin). The DNA synthesized in the complete reaction hybridized to a similar extent to both the leading and the lagging strand templates. These data suggest a role for DNA polymerase δ in leading strand synthesis (22, 27).

The size distribution of the DNA strands synthesized in the complete reconstituted reaction and in the absence of each of the protein components was analyzed by agarose gel electrophoresis (Fig. 2). The products of the complete reaction were quite heterogeneous in length, ranging from a few hundred nucleotides to the length of the input DNA template (2886 nucleotides). DNA synthesis was absolutely dependent upon T antigen, RP-A, DNA polymerase α–primase complex, and the SV40 origin of DNA replication. The reaction was markedly stimulated by topoisomerase I and $PP2A_c$, but neither protein significantly affected the length distribution of newly synthesized strands. The lack of complete dependence upon $PP2A_c$ was expected on the basis of our previous data (15, 16). We previously have demonstrated that replication of covalently closed circular DNA molecules is completely dependent upon topoisomerase I or II in the crude extract or in a partially purified replication system (9, 10). The incomplete requirement for topoisomerase observed in the present experiment may be due to the presence of a small fraction of nicked template molecules that are not topologically constrained. (We have also observed that DNA topoisomerase activity is not required for the replication of linear DNA templates.) In the absence of RF-C, PCNA, or DNA polymerase δ, the amount of DNA synthesis was considerably reduced and the length distribution of newly synthesized strands was heavily weighted toward shorter species. These data are consistent with the results of experiments with model substrates indicating that the processivity of DNA polymerase δ in the presence of RP-A, PCNA, and RF-C is much greater than that of DNA polymerase α–primase complex (28).

Electron Microscopy of Intermediates in the Reconstituted DNA Replication Reaction. To verify that the basic mechanism of DNA replication in the reconstituted system was similar to that operating in the crude system (and *in vivo*), we analyzed the structure of replicative intermediates by electron microscopy. The template used in these experiments was the plasmid pSV5 linearized with the restriction enzyme *Sca* I to provide a convenient reference point within the replicative intermediates. The *Sca* I cleavage site is located approximately 3.7 kilobases (kb) from the SV40 origin in the 8-kb pSV5 plasmid. After 50 min of incubation in the complete reconstituted system, the DNA molecules were isolated and mounted for electron microscopy by the method of Davis *et al.* (24). Replicative intermediates were observed at a frequency of 1–3% relative to the input DNA. The majority of the intermediates contained two replication forks and an internal replication "bubble" (Fig. 3 *A–D*). A small number of intermediates contained only a single replication fork (Fig. 3*E*). These molecules presumably represented cases where one of the two replication forks had progressed to the end of the linear template. In all of the intermediates, two of the branches originating at a common fork were approximately equal in length as expected for newly synthesized daughter duplexes. The extent of replication of each intermediate was calculated, and the population was found to contain molecules ranging from about 10% replicated (e.g., Fig. 3*A*) to about 90% replicated (e.g., Fig. 3*C*). A significant fraction of the newly synthesized daughter duplexes appeared to contain a single-stranded region at one or both of the forks but were otherwise duplex (e.g., Fig. 3*A*). We did observe a small number of molecules (less than 5% of intermediates) that contained a completely single-stranded branch (Fig. 3*F*). The origin of these aberrant molecules is not clear, but they could represent cases where initiation of DNA synthesis occurred on only one of the two parental strands.

To assess the directionality of DNA replication in the reconstituted system, the fractional length of the shorter parental DNA segment was plotted against the fractional extent of replication for each molecule in a randomly chosen population of 52 intermediates (Fig. 4). Due to asynchrony in the movement of the two forks (4), the data points scattered

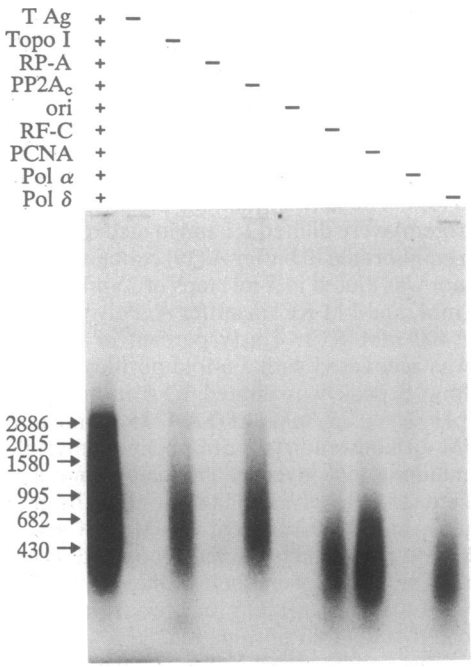

FIG. 2. Protein requirements for SV40 DNA replication. The complete replication reaction contained the components described in Fig. 1, except that pUC.HSO was used as template DNA. Individual proteins were omitted as indicated. In the reaction designated ori⁻, the plasmid pUC9, which lacks the SV40 origin, was used as template. After incubation at 37°C for 90 min, the reaction products were isolated, heat denatured, and subjected to agarose gel electrophoresis (sizes are shown in bp). Topo I, topoisomerase I; Pol α and δ, DNA polymerase α and δ.

Table 1. Strand specificity of DNA replication

	Partial reaction		Complete reaction	
Segment	Lagging	Leading	Lagging	Leading
A (ori)	0.48	0.52	0.40	0.60
B	0.75	0.25	0.46	0.54
C	0.79	0.21	0.43	0.57

Reactions were performed as in Fig. 1 except that they were incubated for 50 min. The table shows the relative fraction of the reaction products that hybridized to the leading and lagging strand templates, respectively. Hybridization probes contained the following segments of the SV40 sequence: A, nucleotides 5092–160; B, nucleotides 294–551; and C, nucleotides 1628–1988. ori, Origin of replication.

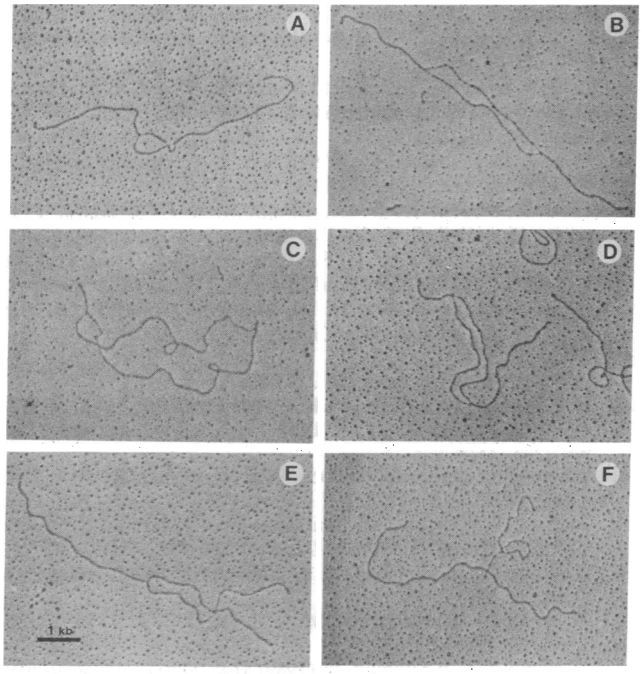

FIG. 3. Electron micrographs of replication intermediates. The 7.9-kb plasmid pSV5 containing the wild-type SV40 origin of DNA replication was linearized with *Sca* I and used as template in the reconstituted replication system. Representative replication intermediates isolated from reconstituted reactions after 50 min of incubation are shown. (Bar = 1 μm.)

about a straight line. The slope of the line obtained by application of the least-squares criterion was −0.56. This result indicates that the average rate of fork movement in the population was about half the average rate of DNA replication. The line extrapolated to an intercept of 0.44 at zero extent of replication, consistent with the location of the cloned SV40 origin at a fractional distance of 0.46 from the end of the linear template. We conclude from these data that DNA replication was initiated near the cloned viral origin of DNA replication and proceeded in a bidirectional fashion. Our electron microscopic data thus provide direct visual evidence that the reconstituted SV40 DNA replication system carries out both leading and lagging strand synthesis at each of two forks moving in opposite directions. Identical results were obtained in a previous study of the replicative intermediates generated in the crude SV40 DNA replication system (4).

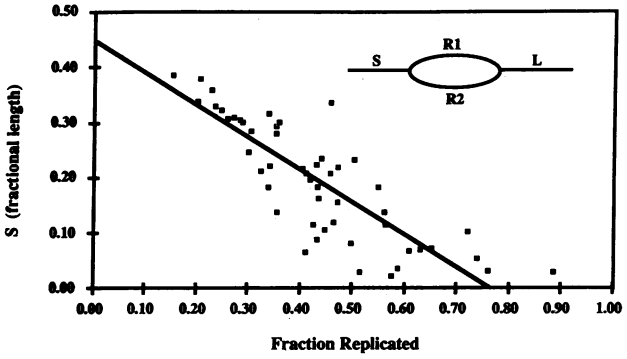

FIG. 4. Origin and direction of DNA replication *in vitro*. Replication intermediates from reaction mixtures containing linearized (*Sca* I) pSV5 DNA template were photographed and measured. The figure summarizes data on the intermediates that contained internal replication bubbles (see Fig. 3 *A–D*). For each molecule the shortest distance from a replication fork to the *Sca* I site (S) was plotted against the extent of replication [(R1 + R2)/2]/[S + L + (R1 + R2)/2]. (All distances were expressed as the fractional length of pSV5.) The line, generated by linear regression analysis, has an x intercept of 0.44 and a slope of −0.56. The *Sca* I site is 0.46 pSV5 map units from the SV40 origin cloned in pSV5.

DISCUSSION

The biochemical studies reported here indicate that efficient replication of duplex DNA templates containing the SV40 origin of replication can be reconstituted with seven highly purified cellular proteins plus the SV40 T antigen. Detailed analysis of the reconstituted system demonstrated that DNA synthesis begins at or near the viral origin and that the intermediates in the reaction contain two active replication forks that move in opposite directions. These characteristics, together with the absolute requirement for T antigen and the wild-type SV40 origin, suggest that DNA replication in the reconstituted replication system closely resembles SV40 DNA replication *in vivo* and in crude HeLa cell extracts (3, 4). The only significant difference between the reconstituted SV40 DNA replication system and the crude SV40 DNA replication system that we have observed so far is that covalently closed daughter molecules are not produced in the reconstituted system. This appears to be due to the fact that the reconstituted system lacks one or more of the enzymes necessary for the processing of RNA primers and joining of the nascent DNA chains. Thus, the daughter molecules contain single-strand interruptions, and the length distribution of newly synthesized chains in the product DNA is quite heterogeneous. The factors required for conversion of the product to covalently closed molecules are present at earlier stages of the purification of DNA polymerase δ but are removed during the final two steps (data not shown). Exonuclease and ligase activities that may be involved in this process have been reported (29).

Based on studies in our laboratory and others, a general picture of the SV40 DNA replication pathway is beginning to emerge (Fig. 5). For purposes of discussion, the reaction can be divided into four major steps: (*i*) binding of T antigen to the origin, (*ii*) local unwinding of the origin, (*iii*) priming and initiation of DNA synthesis, and (*iv*) elongation of nascent

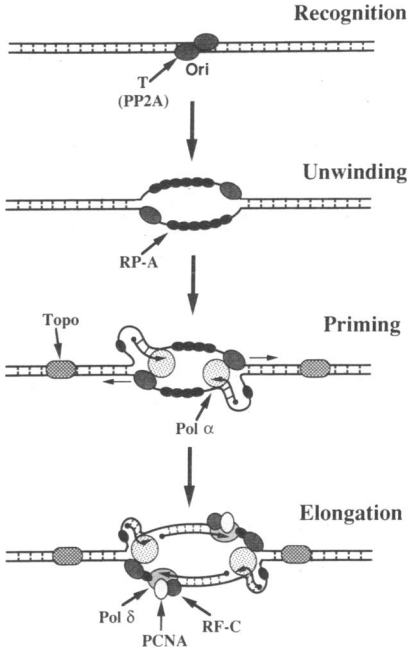

FIG. 5. Model for SV40 DNA replication. See text for details.

chains. It is likely that each of the steps will be further subdivided as additional data accumulates. The initial binding of T antigen to the viral origin appears to be complex. Kinetic analysis indicates that the binding reaction involves at least two discrete steps (D.M.V., A.R., and T.J.K., unpublished data), which lead to the formation of an organized nucleoprotein complex containing as many as 12 T-antigen monomers (30). At physiologic temperatures the formation of this T antigen–origin complex is greatly facilitated by ATP (31, 32), and there is evidence that the bound T antigen perturbs the local structure of the DNA (33, 34). Once the T antigen–origin complex has been assembled, the T antigen catalyzes the unwinding of the duplex in a reaction that requires ATP and RP-A (12, 22, 35) and is stimulated significantly by the dephosphorylation of T antigen by $PP2A_c$ (15, 16). The unwinding reaction is an expression of the intrinsic helicase activity of T antigen which translocates in the 3′-to-5′ direction along the exposed single strands (36, 37). A major role of RP-A in this reaction is to stabilize the unwound strands (9, 12). Following the initial unwinding of the duplex in the origin region, short DNA chains are synthesized as a result of the combined action of the primase and polymerase activities of DNA polymerase α holoenzyme. This enzyme plays a critical role in DNA replication in the reconstituted system because it appears to contain the only activity capable of starting nucleotide chains *de novo*. It is likely that DNA polymerase α interacts in a highly specific way with the other proteins in the initiation complex as suggested in Fig. 5. Efficient and processive elongation of DNA chains additionally requires DNA polymerase δ, DNA topoisomerase I or II, and the accessory proteins PCNA and RF-C. Again, it seems likely that these proteins are incorporated into the nucleoprotein complexes at the forks by specific protein–protein interactions. It has been suggested that DNA polymerase δ serves as the leading strand polymerase, and DNA polymerase α serves as the lagging strand polymerase (17, 23, 37). While this idea is consistent with the available data and has been incorporated into Fig. 5, it should be noted that the evidence supporting it is incomplete. For example, the data are also consistent with the possibility that DNA polymerase δ plays a role in elongation of both the leading and lagging strands. A feature of the chain elongation process not shown in Fig. 4 is the processing of Okazaki fragments on the lagging strand, which involves the removal of RNA primers, the repair of the resulting gaps, and the joining of adjacent nascent strands. The enzymology of these steps has not yet been completely worked out.

The reconstitution of SV40 DNA replication with purified proteins should make it possible to study the detailed interactions and biochemical functions of the component parts of the SV40 replication machinery. The identification of a minimal set of cellular replication proteins should also facilitate the reconstitution of chromosomal DNA replication, once the cellular counterpart(s) of SV40 T antigen and a mammalian origin(s) are identified.

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