THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1991 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 266, No. 3, Issue of January 25, pp. 1942-1949, 1991
Printed in U.S.A.

Purification of DNA Polymerase δ as an Essential Simian Virus 40 DNA Replication Factor*

(Received for publication, July 26, 1990)

Thomas Melendy‡ and Bruce Stillman
From Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

DNA replication from the SV40 origin can be reconstituted *in vitro* using purified SV40 large T antigen, cellular topoisomerases I and II, replication factor A (RF-A), proliferating cell nuclear antigen (PCNA), replication factor C (RF-C), and a phosphocellulose fraction (IIA) made from human cell extracts (S100). Fraction IIA contains all DNA polymerase activity required for replication *in vitro* in addition to other factors. A newly identified factor has been purified from fraction IIA. This factor is required for complete reconstitution of SV40 DNA replication and co-purifies with a PCNA-stimulated DNA polymerase activity. This DNA polymerase activity is sensitive to aphidicolin, but is not inhibited by butylanilinodeoxyadenosine triphosphate or by monoclonal antibodies which block synthesis by DNA polymerase α. The polymerase activity is synergistically stimulated by the combination of RF-A, PCNA, and RF-C in an ATP-dependent manner. Purified calf thymus polymerase δ can fully replace the purified factor in DNA replication assays. We conclude that this factor, required for reconstitution of SV40 DNA replication *in vitro*, corresponds to human DNA polymerase δ.

The DNA replication of eukaryotic chromosomes is a highly regulated event in mammalian cells. Replication occurs only during the S-phase of the cell cycle and is required for cell proliferation. Understanding this central cellular process and its regulation is important not only in elucidating the basic biology of normal cells but also in understanding how normal S-phase controls are circumvented when cells become transformed. The lack of an easily manipulable genetic system for mammalian cells has necessitated a direct biochemical approach to study this problem. This includes the identification of cellular factors required for DNA replication and the analysis of their biochemical function in replication.

In order to identify the factors involved in the replication of mammalian DNA, we have used simian virus 40 (SV40) as a model system (for reviews see Stillman, 1989; DePamphilis and Bradley, 1986; Kelly *et al.*, 1988). The SV40 circular genome has a single origin of replication where bidirectional DNA replication is initiated. In either human or monkey cells, the SV40 large T antigen (TAg)[1] is the only viral protein required for complete viral DNA replication. TAg is a multifunctional protein which is involved in cellular transformation as well as DNA replication. The replication functions provided by TAg include the sequence-specific recognition of the SV40 origin, site-specific ATP-dependent localized melting of the origin and adjacent sequences, and a 3′-5′ helicase/ATPase activity which supports DNA unwinding (Stahl *et al.*, 1986; Dean *et al.*, 1987b; Borowiec and Hurwitz, 1988a, 1988b; reviewed by Borowiec *et al.*, 1990). All other replication functions are provided by host proteins.

A cell-free system has been developed which mimics SV40 replication *in vivo* and demonstrates the same TAg and SV40 origin dependence seen in genetic experiments. Extracts from either monkey or human cells are capable of supporting replication *in vitro* in the presence of exogenously added TAg and plasmid DNA containing the SV40 origin (Li and Kelly, 1984, 1985; Stillman and Gluzman, 1985; Wobbe *et al.*, 1985). This *in vitro* system has proven amenable to biochemical analysis, and fractionation of human cell extracts has identified several proteins that are essential for complete SV40 DNA replication and which are presumably involved in cellular DNA replication *in vivo*. Many lines of evidence had suggested that the DNA polymerase α-primase complex (pol α) is a major replicative polymerase (reviewed by Burgers, 1989). Studies using the SV40 system have corroborated these results (Murakami *et al.*, 1986). In addition, genetic studies in *Saccharomyces cerevisiae* have suggested that topoisomerases I and II are involved in DNA replication (Brill *et al.*, 1987). One or both of these enzymes also appear to be required for SV40 replication *in vitro* from topologically constrained templates (Yang *et al.*, 1987).

Replication factor A (RF-A, also known as RPA and human or HeLa SSB) has been purified from cellular extracts and is a stable, three-polypeptide complex consisting of subunits with molecular masses of 70,000, 34,000 and 11,000 (70, 34, and 11 kDa) (Wobbe *et al.*, 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988). RF-A contains a single-stranded DNA (ssDNA) binding (SSB) activity which resides in the large (70-kDa) subunit (Wold *et al.*, 1989; Brill and Stillman, 1989). In a SV40 origin-specific unwinding assay containing TAg and a topoisomerase, RF-A or a number of other SSB proteins induce the formation of a highly underwound species of DNA (form U) (Wobbe *et al.*, 1987; Dean *et al.*, 1987a; Wold and Kelly, 1988; Tsurimoto *et al.*, 1989; Kenny *et al.*, 1989). The

* This work was supported by United States Public Health Service Research Grant CA13106 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a fellowship from the Damon Runyon-Walter Winchell Cancer Research Fund.

[1] The abbreviations used are: TAg, SV40 large tumor antigen; RF-A, replication factor A; RF-C, replication factor C; PCNA, proliferating cell nuclear antigen; RF-D, replication factor D; pol α, DNA polymerase α-primase complex; pol δ, DNA polymerase δ; hpolδ, human DNA polymerase δ; SSB, single-strand DNA binding protein; BuAdATP, butylanilinodeoxyadenosine triphosphate; BuPdGTP, butylphenyl deoxyguanosine triphosphate; FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate.

appearance of form U is related to an early presynthetic stage of DNA replication in that it appears with the same kinetics of the formation of a DNA·TAg·RF-A complex (Tsurimoto *et al.*, 1989). This complex represents the second of two presynthetic stages seen during SV40 replication *in vitro*. The first stage, which takes 4–5 min, is the ATP-dependent binding of TAg to the SV40 origin sequences and the localized melting of that region (Borowiec and Hurwitz, 1988a, 1988b; Tsurimoto *et al.*, 1989; see Borowiec *et al.*, 1990 for review). The formation of the DNA·TAg·RF-A complex requires an additional 4–5 min of preincubation. Completion of these two stages (8–10 min incubation time) completely eliminates any lag phase in the incorporation of nucleotides upon the addition of all other replication factors (Tsurimoto *et al.*, 1989). Although other SSBs can replace RF-A for unwinding, the requirement for RF-A is very specific in that no other SSB tested is able to replace RF-A for the elongation stages of DNA replication (Wold and Kelly, 1988; Kenny *et al.*, 1989). Indeed, even yeast RF-A (yRF-A), which exhibits the same subunit structure, chromatographic behavior, large subunit ssDNA binding ability, and cell cycle-dependent phosphorylation of the middle (34-kDa/36-kDa) subunit, is incapable of replacing human RF-A in the SV40 DNA replication reaction (Brill and Stillman, 1989; Din *et al.*, 1990).

Proliferating cell nuclear antigen (PCNA) was also purified as a factor required for SV40 replication *in vitro* (Prelich *et al.*, 1987a). It was found that PCNA is identical to the previously described DNA polymerase δ (pol δ) auxiliary factor, which greatly increases the processivity of purified pol δ (Bravo *et al.*, 1987; Prelich *et al.*, 1987b; Tan *et al.*, 1986). Pol δ was initially identified as a novel polymerase activity in rabbit bone marrow extracts (Byrnes *et al.*, 1976). In the absence of PCNA, some DNA is synthesized by the SV40 *in vitro* system; however, the synthesized products are all very short, are lagging strand-specific, and are predominantly derived from regions of DNA surrounding the SV40 origin (Prelich and Stillman, 1988). These results imply that DNA pol δ is required for SV40 DNA replication, and, furthermore, that is is the leading strand DNA polymerase.

Another essential replication protein, replication factor C (RF-C), is a multisubunit complex which was purified based upon its ability to reconstitute SV40 DNA replication *in vitro*. Like PCNA, RF-C is required for synthesis of the leading strand, and, in its absence, lagging strand products are abnormal (Tsurimoto and Stillman, 1989a). Further analysis has identified RF-C as a primer recognition protein with an associated DNA-dependent ATPase activity (Tsurimoto and Stillman, 1990, 1991a, 1991b). The biochemical activities of RF-C are remarkably analogous to those of the protein complex encoded by the bacteriophage T4 genes 44/62 (reviewed by Cha and Alberts, 1989). In addition, PCNA stimulates the RF-C ATPase just as the phage T4 gene 45-encoded protein stimulates the 44/62 protein complex ATPase. These biochemical similarities led to the discovery of limited regions of similarity between the gene 45 and PCNA protein sequences (Tsurimoto and Stillman, 1990). RF-C has been shown to have stimulatory effects on the presumptive replicative DNA polymerases α and δ (Tsurimoto and Stillman, 1989b; Kenny *et al.*, 1989). This effect is greatly enhanced by the combination of the other replication factors RF-A and PCNA (Tsurimoto and Stillman, 1989b).

In the reconstituted SV40 replication system used in this laboratory, in addition to the purified proteins described, there remains only one partially purified fraction that is required for complete *in vitro* replication (Tsurimoto and Stillman, 1989a). This fraction (IIA) is known to contain all required DNA polymerase activities as well as other unknown replication factors. A factor required for reconstitution of SV40 DNA replication has been purified from this fraction and has been shown by several criteria to be equivalent to a human form of DNA polymerase δ.

EXPERIMENTAL PROCEDURES

Materials—[α-^{32}P]dATP and dTTP (800 Ci/mmol) were obtained from Amersham. Phosphocellulose (P-11) and reactive blue 4-agarose were acquired from Whatman and Sigma, respectively. Sephacryl S-300, poly(dA-dT), poly(dA) (average length of 400 nucleotides), and oligo(dT) (average length of 12–15 nucleotides) were purchased from Pharmacia LKB Biotechnology, Inc. The Mono Q HR5/5 and Mono S HR5/5 columns were from Pharmacia and were run on the Pharmacia fast protein liquid chromatography (FPLC) system. Butylanilinodeoxyadenosine triphosphate was a gift from Dr. G. Wright, University of Massachusetts Medical School, Worcester, MA (Lee *et al.*, 1985). Monoclonal antibodies against pol α (SJK 132-20, SJK 287-38, and SJK 237-71) were purified from ascites fluid as described (Harlow and Lane, 1988). Monoclonal cell lines were obtained from the American Type Tissue Culture Collection.

DNA Replication Assays—Reaction conditions for SV40 DNA replication were assayed under conditions as previously described (Stillman and Gluzman, 1985) for 60 min at 37 °C. The plasmid DNA substrates used as templates were pSV010, which contains the entire SV40 genome in pUC18, a total of 7.9 kilobases, and pSV011, which contains the SV40 origin region from *Hin*dIII (nucleotide 5171) to *Sph*I (nucleotide 128) in pUC18 (2.9 kilobases) (Prelich and Stillman, 1988). Incorporation was evaluated as the picomoles of dAMP which remained bound to DEAE-paper (DE81, Whatman) after three washes in 0.5 M Na_2HPO_4. The total volume of the reactions varied from 10 to 50 μl; incorporation levels from all experiments were normalized to reflect incorporation of a 50-μl assay. To analyze the DNA products, the reactions were stopped by addition of a stop solution so that the final mixture contained 0.1 mg/ml predigested pronase (Calbiochem), 20 μg/ml RNase A, 1% (w/v) sodium dodecyl sulfate, and 10 mM Na_2EDTA, and then digested for 30–45 min at 37 °C. The samples were extracted with equal volumes of phenol, then chloroform:isoamyl alcohol (24:1), and then precipitated with ethanol and dissolved in 10 mM Tris-HCl, pH 7.4, and 1 mM Na_2EDTA. Native gel electrophoresis was performed in Tris-borate-EDTA buffer (Maniatis *et al.*, 1982) at 4 V/cm for 8 h. Alkaline gel electrophoresis was performed in 30 mM NaOH and 1 mM Na_2EDTA at 2 V/cm for 12 h. After electrophoresis, agarose gels were fixed in 10% (v/v) methanol, 10% (v/v) acetic acid for 30 min, washed in water for 10 min, and dried onto Whatman No. 3MM paper and autoradiographed.

Replication Factors—SV40 TAg was purified from SF9 cells infected with a recombinant baculovirus expression vector using immunoaffinity chromatography (Simanis and Lane, 1985; Stillman and Gluzman, 1985; Lanford, 1988). Topoisomerases I and II were prepared from calf thymus according to published procedures with slight modifications (Liu and Miller, 1981; Schomburg and Grosse, 1986). PCNA was purified from *Escherichia coli* harboring a plasmid encoding the human PCNA cDNA sequence (Almendral *et al.*, 1987) under control of the bacteriophage T7 promoter.[2] RF-C was purified as described through three steps (second phosphocellulose step) (Tsurimoto and Stillman, 1989a). RF-A was purified to homogeneity as previously reported (Tsurimoto and Stillman, 1989b). Calf thymus DNA polymerase δ (pol δ) was purified through five steps as described (Tsurimoto and Stillman, 1989b; Lee *et al.*, 1984). DNA polymerase α/primase (pol α) was purified from a 293-cell cytosol extract (S100) by immunoaffinity chromatography with an anti-pol α monoclonal antibody (SJK 273-71)-Sepharose as described (Murakami *et al.*, 1986; Tsurimoto and Stillman, 1991a).

Purification of Human Pol δ—The assay used was for reconstitution of SV40 DNA replication *in vitro* (see above). Reactions contained optimized amounts of TAg, calf thymus topoisomerases I and II, RF-A, PCNA, and RF-C (step 4, purified through second phosphocellulose column) in addition to fraction QC (see "Results").

All purification procedures were done at 0–4 °C. Human 293 cells (from 64 liters at 5 × 10^5 cells/ml) were disrupted by Dounce homogenization following swelling of the cells in hypotonic buffer and cytosolic extracts (S100) were prepared as described (Li and Kelly,

[2] K. Fien and B. Stillman, unpublished results.

1984; Stillman and Gluzman, 1985). NaCl was added to the extracts to make the conductivity equal to buffer A (25 mM Tris-HCl, pH 7.5, 1 mM Na_2EDTA, 10% (v/v) glycerol, 0.01% (v/v) Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 mM sodium metabisulfite) containing 0.2 M NaCl. The extract was then applied to a phosphocellulose column (320 ml, 5 × 16.5 cm) equilibrated in buffer A containing 0.2 M NaCl. After washing with 3 column volumes (1 liter) of the same buffer, the column was eluted with buffer A containing 0.33 M NaCl (1 liter) and the peak of protein (as determined by Bradford, 1976) was pooled. This pool was diluted with buffer A until the conductivity reached that of buffer A containing 0.1 M NaCl. This was then applied to a reactive blue 4-agarose column (30 ml, 2.5 × 8.5 cm) equilibrated in buffer A containing 0.1 M NaCl. After washing with 150 ml of the same buffer, the column was step-eluted with buffer A containing 1.0 M NaCl. The protein peak was dialyzed against buffer A containing 0.1 M NaCl and 25% (w/v) sucrose for 8 h.

The dialyzed pool from the reactive blue 4-agarose column was applied to a Sephacryl S-300 column (2.5 × 59 cm) in buffer B (25 mM Tris-HCl, pH 8.0, 1 mM Na_2EDTA, 10% (v/v) glycerol, 0.01% (v/v) Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 mM sodium metabisulfite) containing 0.2 M NaCl. Fractions were assayed for reconstitution of DNA replication and fractions containing the peak of activity were pooled, diluted 1:4 in buffer B, and applied to a Mono Q HR5/5 column. The column was pre-equilibrated in buffer B containing 50 mM NaCl; after loading the sample, the column was washed with 5 ml of the same buffer, and a 12-ml gradient (50–400 mM NaCl) was used to elute the column. Fractions were assayed for reconstitution of DNA replication, and the peak fractions were pooled, taking care to minimize contamination from nearby peaks of protein. The pool from the Mono Q column was either diluted 1:7 with buffer C (35 mM potassium phosphate buffer, pH 7.0, 1 mM Na_2EDTA, 10% (v/v) glycerol, 0.01% (v/v) Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 mM sodium metabisulfite) or dialyzed overnight against buffer C with 50 mM NaCl and 20% (w/v) sucrose. This was then applied to a Mono S HR5/5 column which was sequentially washed with 5 ml of buffer C with 50 mM NaCl, then 2 ml of buffer C with 0.1 M NaCl. The column was developed with a 10-ml linear gradient (0.1–0.5 M NaCl) in the same buffer. Fractions were assayed for reconstitution of DNA replication, and the peak fractions were pooled.

Glycerol gradient sedimentation was performed as described (Melendy and Ray, 1989) in buffer B with 0.1 M NaCl. Fractions were collected from the bottom and assayed for reconstitution of DNA replication and DNA polymerase activity. Aliquots from the fractions were also analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Laemmli, 1970) (10% resolving gel) and silver-stained (Wray *et al.*, 1981).

DNA Polymerase Assays—The DNA polymerase assay used in screening the glycerol gradient fractions contained: poly(dA)/oligo(dT) (19:1; 0.04 mM nucleotides), 40 mM Tris-HCl, pH 6.9, 6 mM $MgCl_2$, 10% (v/v) glycerol, 40 μg/ml acetylated bovine serum albumin, 1 mM dithiothreitol, 0.04 mM dTTP with 1–2 μCi/ml [α-^{32}P]dTTP. Reactions were incubated for 30 min at 37 °C, and incorporation was evaluated as described above for the replication reactions. For reactions using activated poly(dA-dT) the poly(dA-dT) was annealed in 0.2 M KCl, 20 mM Tris-HCl, pH 7.4 at 0.4 mM nucleotide and activated at 0.2 mM nucleotide concentration by the addition of deoxyribonuclease I (0.1 μg/ml) under the conditions described (Maniatis *et al.*, 1982) for 30 min at 30 °C. These assays were performed under conditions described above for the poly(dA)/oligo(dT) assay except that 0.04 mM dATP was added to the reaction and the activated poly(dA-dT) template was used at a final concentration of 0.01 mM nucleotide.

Stimulation of pol δ by other replication proteins on singly primed M13 single-stranded DNA and poly(dA)-oligo(dT) was performed as described (Tsurimoto and Stillman, 1989b) except that 1 mM ATP was added to the primed M13 reactions and poly(dA)-oligo(dT) was used at a final concentration of 4 μM nucleotide.

RESULTS

SV40 Replication Fractions—A modified version of a previously published fractionation scheme for the cytosol extract (S100) from human 293 cells is shown in Fig. 1*A* (Tsurimoto and Stillman, 1989a; Tsurimoto *et al.*, 1989). The only crude fraction essential for reconstitution of DNA replication was fraction IIA. Further fractionation of IIA and subsequent reconstitution of DNA replication has always proven difficult due to the apparent instability of at least one of the replication factors therein. To circumvent this problem, we developed a different method of fractionation of the initial S100 extract. The extract was initially separated on a Q-Sepharose column into a 50 mM NaCl unbound fraction (QFT) and a number of bound fractions from step elutions with 150 mM (QA), 250 mM (QB), 350 mM (QC), and 1 M NaCl (QD) (see Fig. 1*B*). An assay for all the factors present in fraction IIA was used to screen the Q-Sepharose pools. Optimal amounts of TAg, topoisomerases I and II, RF-A, PCNA, and RF-C were included in the assays, which, with the addition of fraction IIA, resulted in complete SV40 DNA replication. As shown in Fig. 2*A*, two fractions were necessary and sufficient to replace fraction IIA for DNA synthesis in this assay; fractions QB and QC. None of the other fractions, QFT, QA, or QD, either alone or in combination, had any appreciable stimulatory effect on the synthesis seen with the QB/QC combination; however, the QA fraction qualitatively altered the distribution of topological forms present in agarose gel analysis of the products (data not shown).

The DNA products of these reactions demonstrated that while neither QB nor QC alone could replace fraction IIA (*lanes 1–3*, Fig. 2*B*), the high level of synthesis produced by the combination of QB and QC yielded monomeric circular products (*lanes 1* and *5*, Fig. 2*B*), which contained discontinuities in the newly synthesized DNA as demonstrated by alkaline agarose gel electrophoresis (data not shown). It was apparent that some additional factor was required for the maturation of these nicked circles into covalently closed topoisomers. The addition of fraction QA to reactions containing fractions QB and QC was able to reconstitute the ladder of covalently closed topoisomers produced in reactions containing fraction IIA (data not shown). However, initiation and elongation did not appear to be affected by the absence of the factor in fraction QA. The origin-specificity of the earliest labeled products (data not shown) indicated that these reactions produce genuine SV40 DNA replication products. The reactions were completely dependent upon a SV40 origin-containing DNA substrate and exogenously added TAg, RF-A, and PCNA. The reactions containing fraction QC were partially dependent upon exogenous RF-C, since fraction QC contains subsaturating levels of RF-C (data not shown). Taken together, these results confirm that the DNA synthesis generated in the QB/QC-containing reactions reflect genuine SV40 DNA replication products.

Purification of Replication Factor D (RF-D)—Replication in the absence of either fraction QB or QC resulted in 10–20-fold lower levels of nucleotide incorporation compared to the complete replication reaction (1.5–4 pmol of dAMP *versus* 45–80 pmol of dAMP incorporated for plasmid SV010 and 20 pmol of dAMP incorporated for plasmid pSV011). Fraction QC contained much higher levels of DNA polymerase activity than fraction QB when assayed on either activated calf thymus DNA or on primed poly(dA) (data not shown). Addition of fraction QC to the reconstituted SV40 replication assay lacking fraction IIA, which contained optimal levels of TAg, topoisomerases I and II, RF-A, PCNA, and RF-C, generated an assay for previously unidentified replication factors common to fractions QB and IIA. Separation of fraction QB on a variety of chromatographic matrices indicated that the essential replication factor(s) in fraction QB behaved as a single chromatographic species which we referred to as replication factor D (RF-D) (data not shown). The replication assay,

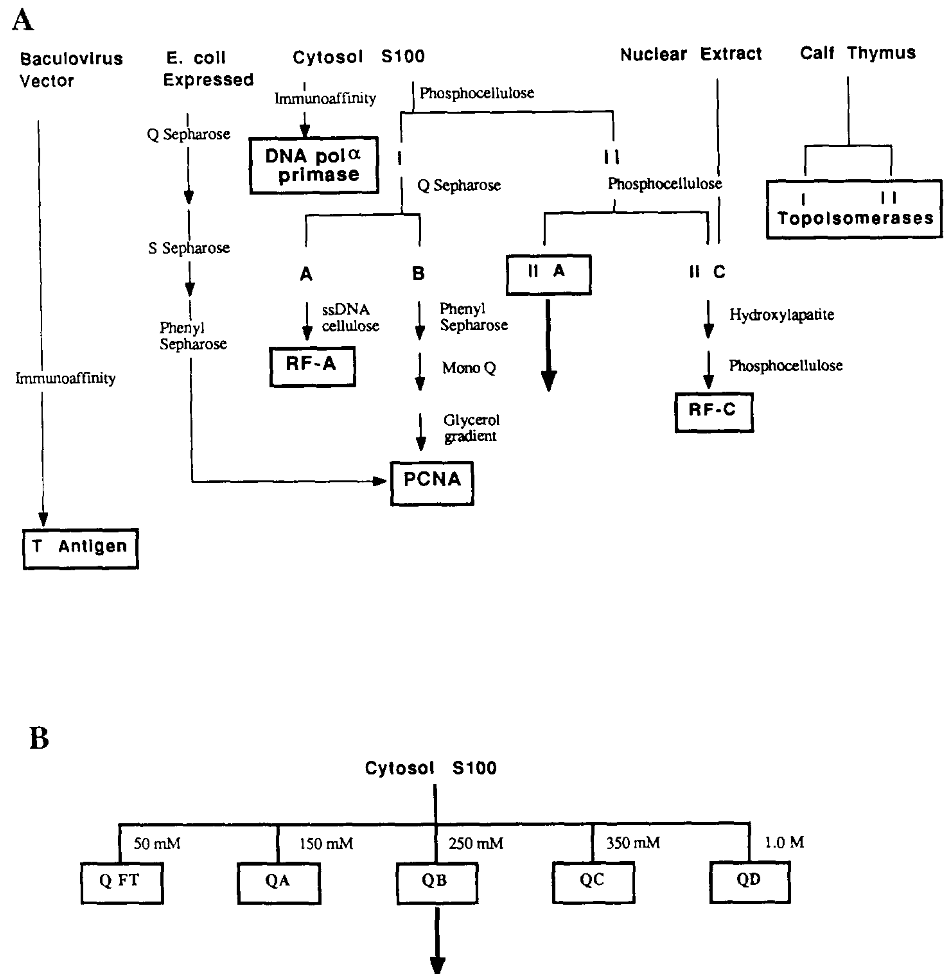

FIG. 1. **Fractionation of components used for reconstitution of SV40 DNA replication.** *A*, purified components used for reconstitution of DNA replication are shown in the *boxes*. Fraction IIA is the only crude fraction required for the SV40 replication reaction and contains pol α/primase as well as pol δ (see below). *B*, S100 was fractionated over a Q-Sepharose column as described under "Experimental Procedures." Of the resultant five fractions, only two, QB and QC, were required to replace fraction IIA (above) for DNA synthesis in the reconstituted replication reaction. Fixed amounts of fraction QC were used to assay for essential factors common to fraction QB and fraction IIA.

modified by replacing fraction QC for fraction IIA, was used as a criterion for the purification of RF-D.

To ensure that a factor from fraction IIA was being purified and because the phosphocellulose fractionation of S100 into IIA generated a rapid 10-fold decrease in protein, fraction IIA was used as the starting material for the first step in the purification. Reactive blue 4-agarose separation had resulted in nearly quantitative recovery of RF-D from the QB fraction and had generated a 5-fold purification. Although not as selective when using fraction IIA as the starting material, this step was very rapid and resulted in a 20-fold concentration of the extract in preparation for Sephacryl S-300 gel filtration chromatography. Further purification on the Pharmacia FPLC Mono Q and Mono S matrices resulted in a fraction with a high concentration of RF-D activity which completely replaced the QB fraction in SV40 DNA replication assays (Fig. 2*B*, *lane 6*).

Identification of RF-D as Polymerase δ—Results of a glycerol gradient analysis of RF-D are shown in Fig. 3*A*. Fractions were assayed as described above for the reconstitution of *in vitro* replication. RF-D sedimented as a single peak in fractions 10 and 11. The same fractions were also assayed for DNA polymerase activity on a poly(dA)-oligo(dT) template both in the presence and absence of PCNA. As shown in Fig. 3*A*, a PCNA-stimulated polymerase activity co-sedimented precisely with RF-D activity. A potent nuclease which sedimented more slowly in the glycerol gradient prevented detection of weak exonuclease activity in the polymerase-containing fractions (data not shown).

Although the polyacrylamide gel electrophoresis showed that several polypeptides were still present in the glycerol gradient fractions, a 130-kDa band (indicated by the *arrow*, Fig. 3*B*) may correspond to the peak of RF-D activity. This band is similar in size to that of the catalytic subunit of pol δ from calf thymus (125-kDa) and *S. cerevisiae* and the predicted size of the yeast polIII (ypolδ) gene product (124-kDa) (Lee *et al.*, 1984; Boulet *et al.*, 1989). A smaller 50-kDa subunit found in pol δ preparations from both calf thymus and *S. cerevisiae* was not observed in this preparation. A 50-kDa band might have been present in these fractions but may have been undetectable due to the low levels of enzyme present. In addition, polymerase catalytic subunit labeling experiments on the RF-D preparation indicated a labeled band at approximately 130 kDa (data not shown).

Pol δ is distinguished by its response to several inhibitors. Like pol α, pol δ is inhibited by aphidicolin; however, pol α and pol δ are differentially inhibited by butylanilinodeoxyadenosine triphosphate (BuAdATP) and butylphenyl deoxyguanosine triphosphate (BuPdGTP) and by monoclonal antibodies which neutralize pol α activity (Byrnes, 1985; Syväoja *et al.*, 1990). The DNA polymerase activity of RF-D was sensitive to aphidicolin at levels similar to levels which inhibit immunopurified human pol α (data not shown). However, this polymerase activity was much more resistant to inhibition by BuAdATP than was purified pol α (Fig. 4*A*). Fig. 4 indicates that the RF-D DNA polymerase was only slightly inhibited by concentrations of BuAdATP 3 orders of magnitude higher than those required to inhibit pol α by 50%. Calf thymus pol

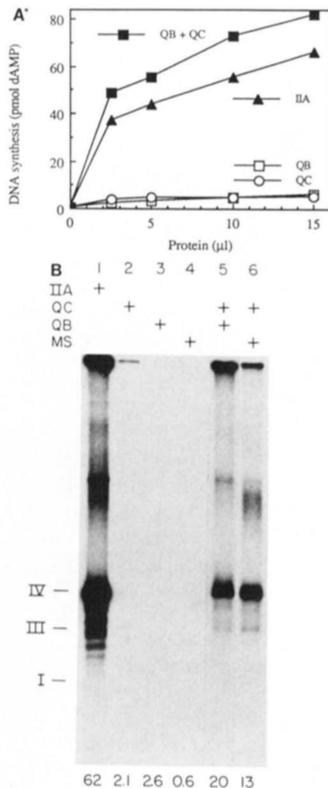

FIG. 2. **Replacement of fraction IIA with Q-Sepharose fractions QB and QC for complete SV40 DNA replication.** *A*, titration of increasing amounts of either fraction IIA (6.5 mg/ml) (*filled triangles*), fraction QC (5.7 mg/ml) (*circles*), fraction QB (6.7 mg/ml) (*squares*), or fraction QB with a constant amount (5 μl, 29 μg) of fraction QC (*filled squares*) into the replication reactions containing optimized levels of TAg, both topoisomerases, RF-A, PCNA, and RF-C. DNA synthesis is expressed as picomoles of dAMP incorporated in 60 min. *B*, gel analysis of SV40 DNA replication products. Reactions contained optimal amounts of TAg, topoisomerases, RF-A, PCNA, and RF-C as described under "Experimental Procedures." To these reactions were added fraction IIA (to a final concentration of 1.56 mg/ml), fraction QB (1.34 mg/ml), fraction QC (0.58 mg/ml), or the Mono S Pool 6 (*MS*) from the fractionation (16.8 μg/ml), as indicated. Reactions products were isolated and an equal proportion of each was separated in a 0.8% agarose gel at 2.5 V/cm for 10 h. After electrophoresis, the gel was fixed, dried, and autoradiographed. The positions of form I and form II of the template plasmid (pSV011) are indicated. The amount of DNA synthesis (picomoles of dAMP incorporated) is shown at the *bottom* of each *lane*.

α and pol δ displayed similar differences in sensitivity to BuAdATP.[3] In addition, monoclonal antibodies which neutralized pol α activity had no effect on the RF-D polymerase activity (Fig. 4*B*). Therefore, the RF-D polymerase activity behaved in a manner indistinguishable from pol δ with all inhibitors tested.

It has been demonstrated that calf thymus pol δ is stimulated by the human replication factors RF-A, PCNA, and RF-C in a synergistic manner (Tsurimoto and Stillman, 1989b). Fig. 5*A* shows the effect of these three replication factors on the RF-D DNA polymerase activity when a single primed M13 single-stranded DNA template was used. The products were isolated from these reactions, denatured, and subjected to denaturing gel electrophoresis in an alkaline agarose gel. RF-A and RF-C had little effect on the RF-D DNA polymerase activity (Fig. 5*A*, *lanes 1–3*). There was a slight increase in processivity seen when PCNA was added to the RF-D DNA

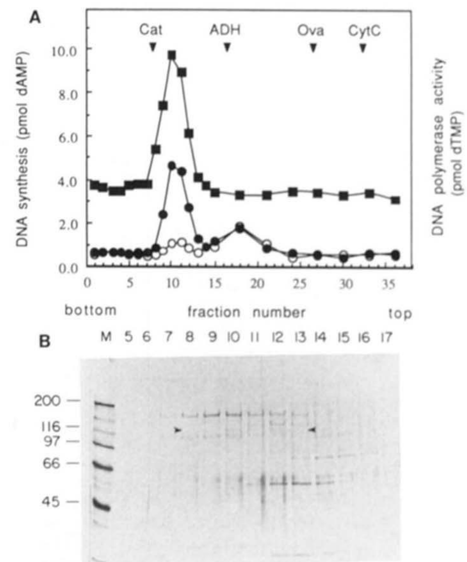

FIG. 3. **Co-sedimentation of replication activity with a PCNA-stimulated DNA polymerase in a glycerol gradient.** Centrifugation was as described under "Experimental Procedures." *A*, fractions (10 μl) were assayed for reconstitution of SV40 DNA replication (*filled squares*) and for polymerase activity on primed poly(dA) in the absence (*circles*) and presence (*filled circles*) of PCNA (10 μg/ml). Sedimentation of marker proteins: catalase (232 kDa), yeast alcohol dehydrogenase (150 kDa), ovalbumin (43 kDa), and cytochrome *c* (12 kDa) are indicated. *B*, equal volume samples of each fraction indicated were subjected to SDS-polyacrylamide gel electrophoresis and silver-stained as described under "Experimental Procedures." The *lane number* corresponds to the fractions in *A*. Positions of molecular mass markers are indicated. A protein of 130 kDa is indicated by the *arrow*.

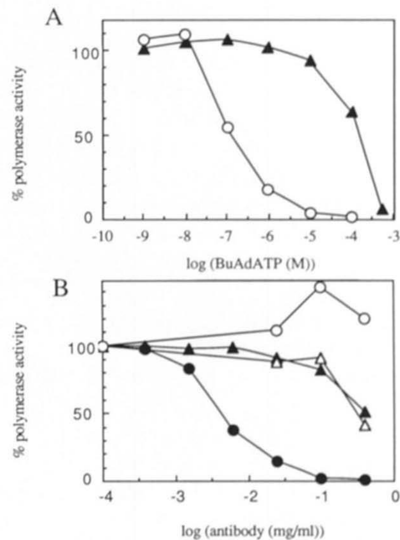

FIG. 4. **Inhibition of human DNA polymerase δ by BuAdATP and monoclonal antibodies against human DNA polymerase α.** *A*, polymerase reactions using activated poly(dA-dT) were performed as described under "Experimental Procedures" with increasing levels of the inhibitor BuAdATP. The percent of pol δ (*filled triangles*) and pol α (*circles*) activity remaining at each concentration was plotted. *B*, monoclonal antibodies against human pol α which both do (SJK132-20, *filled symbols*) and do not (SJK 273-71, *open symbols*) neutralize pol α activity were preincubated with equal amounts of pol δ (*triangles*) or pol α (*circles*) for 45 min on ice. Polymerase reactions using a primed poly(dA) template (and PCNA at 10 μg/ml in the pol δ reactions) were then added, and the reactions were incubated and analyzed as described under "Experimental Procedures." Pol δ fraction was from the Mono S step of the purification.

[3] A. So, personal communication.

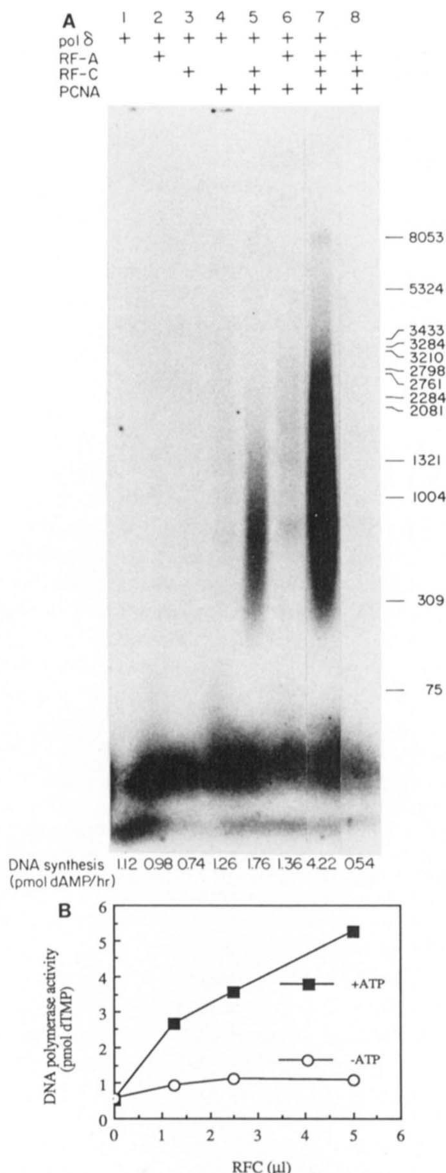

FIG. 5. **Synergistic stimulation of polymerase δ by RF-A, PCNA, and RF-C is ATP-dependent.** *A,* equal amounts of pol δ (Mono S step, Pool 6, 16.8 ng/μl) were incubated with RF-A (16 ng/μl), PCNA (8.4 ng/μl), or RF-C (88 ng/μl, second phosphocellulose pool (Tsurimoto and Stillman, 1989a)) with singly primed, single-stranded M13 DNA as a template. The products were isolated and subjected to electrophoresis in an alkaline agarose gel as described under "Experimental Procedures." Migration of markers, given in number of bases, are indicated on the *right. B,* reactions containing RF-D DNA polymerase (Mono S step, Pool 6, 16.8 ng/μl), RF-A (16 ng/μl), PCNA (8.4 ng/μl), and primed poly(dA) (4 μM) were incubated with varying amounts of RF-C (2.2 mg/ml) in the presence (*filled squares*) or absence (*circles*) of ATP (1 mM). DNA synthesis was measured by incorporation of dAMP into DNA.

polymerase (Fig. 5*A, lanes 1* and *4*). However, it should be noted that the stimulation by PCNA is very small in this experiment due to the difference in pH and template DNA from that used in the experiments in Fig. 3*A*. Pol δ is strongly stimulated by PCNA alone at pH 6.9 (Fig. 3*A*), while at pH 7.5, this effect is difficult to detect.[4] The combination of either PCNA plus RF-C or PCNA plus RF-A both resulted in a slightly larger stimulation of RF-D DNA polymerase activity than each factor alone; however, the combination of RF-A, RF-C, and PCNA stimulated the RF-D DNA polymerase activity in a greater than additive manner (Fig. 5*A, lanes 5–7*). The combination appeared to affect the processivity of the enzyme as well as the number of primed events. To further demonstrate that this was a specific effect, it was shown that the stimulation of RF-D DNA polymerase by RF-C in the presence of RF-A and PCNA was ATP-dependent (Fig. 5*B*). This was to be expected since RF-C has an intrinsic ATPase activity that is required for its stimulatory activity (Tsurimoto and Stillman, 1991a and 1991b).

As a final verification that the replication activity was equivalent to pol δ, we replaced the RF-D fraction with highly purified calf thymus pol δ in a reconstituted replication reaction. Both the RF-D polymerase and highly purified calf thymus pol δ were titrated into replication reactions identical with those used to assay the purification of this factor. As can be seen in Fig. 6, calf thymus pol δ completely replaced the RF-D polymerase in the replication reaction. The reaction products were virtually indistinguishable from those shown in Fig. 2 (*lane 6*) and demonstrated both origin specificity of the earliest synthesized fragments and bidirectionality of replication (Tsurimoto *et al.*, 1990).

DISCUSSION

There are several ways in which to study a complex *in vitro* system. The approach used in this study has been to subdivide the required extract and then add the subfractions back to the reaction to determine which of the subfractions are necessary to reconstitute replication. This method has been used in this laboratory to isolate the SV40 cellular replication factors RF-A, PCNA, and RF-C. Fraction IIA, the only crude fraction still required to reconstitute SV40 DNA replication *in vitro*, was known to contain DNA pol α/primase and all other factors required to reconstitute DNA replication. Using the method of purification and reconstitution, a factor required to reconstitute DNA replication was isolated from fraction IIA. This factor co-purified with a DNA pol δ activity.

The criteria used to verify that this factor is pol δ were severalfold. Pol δ was initially identified as an aphidicolin-sensitive polymerase activity which co-purified with an exonuclease activity (Byrnes *et al.*, 1976; Lee *et al.*, 1984). Since that time it has been found that pol δ is more resistant to inhibition by BuAdATP/BuPdGTP than is pol α, and pol δ is not inhibited by monoclonal antibodies which block pol α activity (Byrnes, 1985; Syväoja *et al.*, 1990).[3] The most stringent criterion available in identifying pol δ is that pol δ is strongly stimulated by PCNA through very specific interactions (Tan *et al.*, 1986; Bauer and Burgers, 1988). More recently it was shown that under different conditions, the

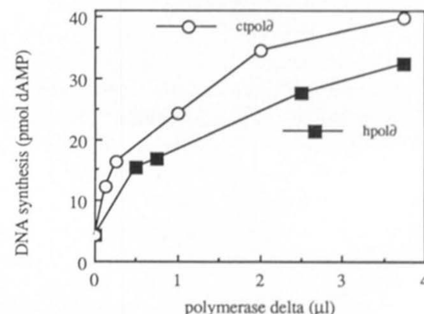

FIG. 6. **Replacement of human pol δ with calf thymus pol δ in *in vitro* SV40 replication.** Human pol δ (*hpolδ*) (*filled squares*) (Mono S fraction, Pool 6, 0.42 mg/ml) or calf thymus pol δ (*ctpolδ*) (*circles*) (0.04 mg/ml) was titrated into replication reactions containing RF-A, PCNA, RF-C, TAg, topoisomerases, and fraction QC as described above.

[4] T. Tsurimoto, personal communication.

combination of RF-A, PCNA, and RF-C have a highly specific and greater than additive stimulatory effect on pol δ (Tsurimoto and Stillman, 1989b). The polymerase which we have purified has met all of these criteria and as such we conclude that the essential replication factor RF-D is human polymerase δ.

The finding that PCNA is a required replication factor and that it is equivalent to a pol δ auxiliary protein implicated pol δ in DNA replication. It has also been demonstrated that RF-C, another factor required for the reconstitution of SV40 DNA replication, also has specific interactions with pol δ. In fact, the combination of RF-C and PCNA, as a primer recognition complex, and RF-A, seem to have a very specific effect both on pol α and pol δ. These three factors combine to prevent pol α from using recessed 3′ ends of double-stranded DNA as a primer while simultaneously stimulating the initiation of pol δ on the same 3′ ends (Tsurimoto and Stillman, 1991a, 1991b). In addition to these data, several laboratories have published other evidence that pol δ may be required in DNA replication.

Weinberg and Kelly (1989) demonstrated that they could reconstitute SV40 DNA replication with multiple components from human cells including a chromatographic fraction that contained a PCNA-stimulated DNA polymerase. Highly purified calf thymus pol δ, however, could not replace this fraction in the replication reaction. We, and others, have demonstrated that calf thymus pol δ can replace human pol δ in the SV40 system (this paper; Tsurimoto *et al.*, 1990; Lee *et al.*, 1989). This discrepancy is most likely explained by the absolute requirement of RF-C for reconstitution of DNA replication. Similarities in sedimentation and chromatographic behavior between RF-C and human pol δ make it likely that both RF-C and pol δ were present in their most purified human pol δ-containing fraction.

There is now a wealth of evidence supporting the contention that pol δ is essential for SV40 DNA replication *in vitro*. The initial evidence was the finding that PCNA, a processivity factor for pol δ, was required to reconstitute DNA replication. Further, RF-C, also required for DNA replication *in vitro*, stimulates pol δ in conjunction with the other replication factors, RF-A and PCNA. It has now been shown that PCNA and RF-C form a primer recognition complex, which, in cooperation with RF-A, regulates which polymerases recognize DNA primers. Whereas primer recognition by pol α is inhibited by this regulation, pol δ is strongly stimulated by the actions of these replication factors (see Tsurimoto and Stillman, 1991a, 1991b). More direct evidence is presented in this paper and by Lee *et al.* (1989) that pol δ is the essential factor present in fractions required for reconstitution of DNA replication.

Pol δ is presumably involved in replication of chromosomal DNA *in vivo*. Drug and monoclonal antibody inhibition studies of permeabilized cells have demonstrated differential inhibition of different stages of DNA replication indicating that more than one polymerase is involved in DNA replication. Both pol α and pol δ are sensitive to aphidicolin, and aphidicolin inhibits different stages of replication at different levels indicating a role for multiple aphidicolin-sensitive DNA polymerases (Decker *et al.*, 1986). Partial resistance of DNA replication to the inhibitor BuPdGTP and to the pol α-neutralizing monoclonal antibody, SJK-132, also implicates a DNA polymerase besides pol α in DNA replication (Dresler and Frattini, 1986; Hammond *et al.*, 1987). Since pol δ is sensitive to aphidicolin, resistant to higher levels of BuPdGTP than pol α, and is unaffected by monoclonal antibody SJK-132, it is the likely candidate for the second DNA polymerase required for DNA replication *in vivo*. Genetic studies also support the dual polymerase hypothesis. Biochemical and genetic analyses of yeast PCNA and yeast pol δ (*CDC2*) demonstrated that the gene products are essential for progression through S phase and DNA replication in *S. cerevisiae* (Bauer and Burgers, 1988; Boulet *et al.*, 1989; Sitney *et al.*, 1989). *In vivo* studies using both genetic data and drug inhibition of replicative DNA polymerases support the view that pol δ is required for cellular DNA replication.

Since we now know that fraction IIA contains both pol α and pol δ, which have both been shown to be required for replication, what other factors within fraction IIA are still required for DNA replication? The easiest factors to identify are those which affect either initiation or elongation. To answer this question, immunopurified pol α/primase was titrated into replication reactions using all the purified components (TAg, topoisomerases, RF-A, PCNA, RF-C, and human pol δ) but without fraction QC. Immunopurified pol α was able to completely replace fraction QC in the reconstituted replication reaction (data not shown; Tsurimoto *et al.*, 1990). In this purified system, pol α replicated the lagging strand, whereas pol δ replicated the continuously synthesized leading strand. The products of this purified system, however, still contained gaps on the lagging strand because the Okazaki fragments were not ligated together (Tsurimoto *et al.*, 1990). The discontinuous nature of the replication products from this purified system is a result of the lack of one or more "maturation" activities which are present in fraction IIA. Presumably one or all of these activities are present in fraction QA since fraction QA, in conjunction with fractions QB and QC, can generate completely replicated covalently closed topoisomers. Such activities must include exonuclease, RNase, and ligase activities (Ishimi *et al.*, 1988). Additional activities which might be involved include repair-type polymerase activities or factors which stimulate known replication proteins. However, apparently the only cellular factors that are essential for initiation and elongation of SV40 DNA replication *in vitro* are RF-A, PCNA, RF-C, pol α/primase, pol δ, and topoisomerases I and II (Tsurimoto *et al.*, 1990).

The requirement of two polymerases in the minimal reconstituted DNA replication system leads to questions concerning the mechanism behind initiation and elongation. Since the primase associated with pol α is the only source of priming, it is evident that pol α/primase must be the first factor to initiate actual nucleic acid (and presumably DNA) synthesis. Reaction products generated by replication reactions without PCNA, the pol δ auxiliary factor, demonstrated that PCNA, and pol δ by implication, are required for leading strand synthesis (Prelich and Stillman, 1988). Hence, pol δ must be primed by a DNA strand synthesized by pol α/primase. In the accompanying manuscripts (Tsurimoto and Stillman, 1991a, 1991b), a mechanism for the process of switching from pol α to pol δ synthesis is described. RF-C and PCNA form a complex on the 3′ end of the newly synthesized strand. This complex blocks pol α recognition of the primer but stimulates primer recognition by pol δ. RF-A assists in this process, possibly by preventing polymerase and RF-C·PCNA binding to nearby single-stranded regions of DNA. The effect is that pol α/primase synthesizes the initial RNA/DNA fragment on each strand of the origin. The 3′ ends are then blocked from further pol α extension by RF-C·PCNA assisted by RF-A. Pol α/primase then proceeds as the lagging strand polymerase. The primer recognition complex is recognized by pol δ which then uses these primers to begin leading strand synthesis, with RF-C and PCNA as a leading strand complex, on both template strands. Surprisingly, this leading strand complex

(pol δ, RF-C, and PCNA) can be replaced by similar leading strand complexes from either bacteriophage T4 (gene 43, 45, and 44/66 products) or bacteriophage T7 (T7 polymerase and thioredoxin), but not with the *E. coli* DNA polymerase III holoenzyme (Tsurimoto *et al.*, 1990). The system of purification and reconstitution of DNA replication has elucidated much about the factors and mechanisms involved in the early and elongation stages of DNA replication. Now that this purified system is available for study, much more can be done to elucidate the mechanisms of eukaryotic DNA replication. Additionally, studies can now be initiated on how DNA replication and the replication factors are controlled.

Acknowledgments—We thank S. Longinotti and N. Kessler for technical assistance and T. Tsurimoto and J. Diffley for scientific discussions and advice. We also thank J. Duffy for the artwork.

REFERENCES

Almendral, I. M., Heubsch, D., Blundell, P. A., MacDonald-Bravo, H., and Bravo, R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 1575-1579

Bauer, G. A., and Burgers, P. M. J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7506-7510

Borowiec, J. A., and Hurwitz, J. (1988a) *EMBO J.* **7,** 3149-3158

Borowiec, J. A., and Hurwitz, J. (1988b) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 64-68

Borowiec, J. A., Dean, F. B., Bullock, P. A., and Hurwitz, J. (1990) *Cell* **62,** 181-184

Boulet, A., Simon, M., Faye, G., Bauer, G. A., and Burgers, P. M. J. (1989) *EMBO J.* **8,** 1849-1854

Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248-254

Bravo, R., Frank, R., Blundell, P. A., and MacDonald-Bravo, H. (1987) *Nature* **326,** 515-517

Brill, S. J., and Stillman, B. (1989) *Nature* **342,** 92-95

Brill, S. J., DiNardo, S., Voelkel-Meiman, K., and Sternglanz, R. (1987) *Nature* **326,** 414-416

Burgers, P. M. J. (1989) *Prog. Nucleic Acid Res. Mol. Biol.* **37,** 235-280

Byrnes, J. J. (1985) *Biochem. Biophys. Res. Commun.* **132,** 628-634

Byrnes, J. J., Downey, K. M., Black, V. L., and So, A. G., (1976) *Biochemistry* **15,** 2817-2823

Cha, T.-A., and Alberts, B. M. (1989) *J. Biol. Chem.* **264,** 12220-12225

Dean, F. B., Bullock, P., Murakami, Y., Wobbe, C. R., Weissbach, L., and Hurwitz, J. (1987a) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 16-20

Dean, F. B., Dodson, M., Echols, H., and Hurwitz, J. (1987b) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8981-8985

Decker, R. S., Masamitsu, Y., Possenti, R., and DePamphilis, M. L. (1986) *Mol. Cell. Biol.* **6,** 3815-3825

DePamphilis, M. L., and Bradley, M. K. (1986) *The Papovaviriadae* (Salzman, N. P., ed) pp. 99-246, Plenum Publishing Co., New York

Din, S., Brill, S. J., Fairman, M. P., and Stillman, B. (1990) *Genes & Dev.* **4,** 968-977

Dresler, S. L., and Frattini, M. G. (1986) *Nucleic Acids Res.* **14,** 7093-7102

Fairman, M. P., and Stillman, B. (1988) *EMBO J.* **7,** 1211-1218

Hammond, R. A., Byrnes, J. J., and Miller, M. R. (1987) *Biochemistry* **26,** 6817-6824

Harlow, E., and Lane, D. (eds) (1988) *Antibodies: A Laboratory Manual,* p. 311, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Ishimi, Y., Claude, A., Bullock, P., and Hurwitz, J. (1988) *J. Biol. Chem.* **263,** 19723-19733

Kelly, T. J., Wold, M. S., and Li, J. (1988) *Adv. Virus Res.* **34,** 1-42

Kenny, M. K., Lee, S.-H., and Hurwitz, J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 9757-9761

Laemmli, U. K. (1970) *Nature* **227,** 680-685

Lanford, R. E. (1988) *Virology* **167,** 72-81

Lee, M. Y. W. T., Tan, C.-K., Downey, K. M., and So, A. G. (1984) *Biochemistry* **23,** 1906-1913

Lee, M. Y. W. T., Toomey, N. L., and Wright, G. E. (1985) *Nucleic Acids Res.* **13,** 8623-8630

Lee, S.-H., Eki, T., and Hurwitz, J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 7361-7365

Li, J. J., and Kelly, T. J. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 6973-6977

Li, J. J., and Kelly, T. J. (1985) *Mol. Cell. Biol.* **5,** 1238-1246

Liu, L. F., and Miller, K. G. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 3487-3491

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Melendy, T. E., and Ray, D. S. (1989) *J. Biol. Chem.* **264,** 1870-1876

Murakami, Y., Wobbe, C. R., Weissbach, L., Dean, F. B., and Hurwitz, J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 2869-2873

Prelich, G., and Stillman, B. (1988) *Cell* **53,** 117-126

Prelich, G., Kostura, M., Marshak, D. R., Mathews, M. B., and Stillman, B. (1987a) *Nature* **326,** 471-475

Prelich, G., Tan, C. K., Kostura, M., Mathews, M. B. So, A. G., Downey, K. M., and Stillman, B. (1987b) *Nature* **326,** 517-520

Schomburg, V., and Grosse, F. (1986) *Eur. J. Biochem.* **160,** 451-457

Simanis, V., and Lane, D. P. (1985) *Virology* **144,** 88-100

Sitney, K. C., Budd, M. E., and Campbell, J. L. (1989) *Cell* **56,** 599-605

Stahl, G., Dröge, P., and Knippers, R. (1986) *EMBO J.* **5,** 1939-1944

Stillman, B. (1989) *Annu. Rev. Cell Biol.* **5,** 197-245

Stillman, B., and Gluzman, Y. (1985) *Mol. Cell. Biol.* **5,** 2051-2060

Syväoja, J., Suomensaari, S., Nishida, C., Goldsmith, J. S., Chui, G. S. J., Jain, S., and Linn, S. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6664-6668

Tan, C.-K., Castillo, C., So, A. G., and Downey, K. M. (1986) *J. Biol. Chem.* **261,** 12310-12316

Tsurimoto, T., and Stillman, B. (1989a) *Mol. Cell. Biol.* **9,** 609-619

Tsurimoto, T., and Stillman, B. (1989b) *EMBO J.* **8,** 3883-3889

Tsurimoto, T., and Stillman, B. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1023-1027

Tsurimoto, T., and Stillman, B. (1991a) *J. Biol. Chem.* **266,** 1950-1960

Tsurimoto, T., and Stillman, B. (1991b) *J. Biol. Chem.* **266,** 1961-1968

Tsurimoto, T., Fairman, M. P., and Stillman, B. (1989) *Mol. Cell. Biol.* **9,** 3839-3849

Tsurimoto, T., Melendy, T. E., and Stillman, B. (1990) *Nature* **346,** 534-539

Weinberg, D. H., and Kelly, T. J., (1989) *Proc. Natl. Sci. U. S. A.* **86,** 9742-7946

Wobbe, C. R., Dean, F., Weissbach, L., and Hurwitz, J. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 5710-5714

Wobbe, C. R., Weissbach, L., Borowiec, J. A., Dean, F., Murakami, Y., Bullock, P., and Hurwitz, J. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 1834-1838

Wold, M. S., and Kelly, T. J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 2523-2527

Wold, M. S., Weinberg, D. H., Virshup, D. M., Li, J. J., and Kelly, T. J. (1989) *J. Biol. Chem.* **264,** 2801-2809

Wray, W., Boulikas, T., Wray, V. P., and Hancock, R. (1981) *Anal. Biochem.* **118,** 197-203

Yang, L., Wold, M. S., Li, J. J., Kelly, T. J., and Liu, L. F. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 950-954