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Biochemistry

Requirement for two DNA polymerases in the replication of simian virus 40 DNA *in vitro*

(DNA polymerase α/DNA polymerase δ/eukaryotic DNA replication)

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ABSTRACT **DNA polymerase α–primase has long been considered the primary, if not sole, replicative DNA polymerase in eukaryotic cells. However, recent experiments have provided indirect evidence that a second DNA polymerase may play a role in DNA replication. To identify cellular proteins necessary for DNA synthesis in mammalian cells, we have been studying the cell-free system developed for the replication of simian virus 40 DNA. In this report, we present direct evidence that a second DNA polymerase is required in addition to DNA polymerase α–primase complex to obtain efficient replication of simian virus 40 origin-containing DNA. This DNA polymerase activity is not affected by monoclonal antibodies that inhibit the activity of DNA polymerase α and is relatively resistant to the inhibitor [N^2-(*p-n*-butylphenyl)-9-(2-deoxy-β-D-ribofuranosyl)guanine 5′-triphosphate]. Moreover, the activity of the polymerase is highly dependent upon the accessory protein, proliferating-cell nuclear antigen. These characteristics are consistent with the hypothesis that this second DNA polymerase is DNA polymerase δ.**

To understand the mechanisms involved in the replication of cellular chromosomes, it is essential to identify and characterize the cellular proteins involved in the process. Since no cell-free system for directly examining the replication of cellular chromosomes exists at the present time, we have been exploiting a system developed in this laboratory (1) that catalyzes the replication of the papovavirus simian virus 40 (SV40) *in vitro*. Detailed analysis of this system indicates that it closely reflects the requirements for SV40 DNA replication *in vivo* (2). Replication requires a functional SV40 origin sequence and the viral initiator protein, tumor antigen (T antigen). Replication also requires a number of cellular proteins that are present in soluble extracts from permissive host cells. Since T antigen is the only viral protein required for DNA replication, the remainder of the viral replication machinery must be supplied by the host cells. It follows that insights obtained from the study of SV40 DNA replication should also provide useful information about cellular DNA replication.

Our approach to the identification of cellular proteins required for the replication of SV40 DNA has been to fractionate the extract required for the reaction and to determine which fractions are necessary to reconstitute T-antigen-dependent replication of plasmids containing the SV40 replication origin. In this manner we have demonstrated that a minimum of seven cellular proteins are required for efficient viral DNA replication *in vitro* (3). Six of these proteins have been purified to near homogeneity in this and other laboratories (3–10).

One of the cellular components required for SV40 DNA replication in the reconstituted cell-free system is the DNA polymerase α–primase complex. A large body of literature has accumulated implicating DNA polymerase α as the major, if not the only, replicative DNA polymerase in mammalian cells (for a review, see ref. 11). However, recent experiments have provided indirect evidence that a second DNA polymerase, designated DNA polymerase δ, may be involved in SV40 DNA replication (and by implication cellular DNA replication). DNA polymerase δ was originally identified as a polymerase activity in calf thymus that possessed chromatographic and biochemical properties distinct from DNA polymerase α (12). A role for this polymerase in SV40 DNA replication was suggested by the finding that proliferating-cell nuclear antigen (PCNA), a 37-kDa protein that is absolutely required for viral DNA replication *in vitro* (10), is identical to a factor that greatly stimulates the activity of DNA polymerase δ (13–15). Since omission of PCNA from the SV40 DNA replication system leads to the synthesis of short nascent chains that are derived predominantly from the lagging strand (3, 16), it has been suggested that DNA polymerase α–primase complex performs lagging-strand synthesis, and DNA polymerase δ performs leading-strand synthesis (17). However, no direct evidence to support this model has been reported.

In this report we describe the further fractionation of the SV40 cell-free system. We present evidence that both DNA polymerase α–primase complex and a second DNA polymerase activity are required for SV40 DNA replication. The properties of the second polymerase activity are consistent with the hypothesis that it is human DNA polymerase δ.

MATERIALS AND METHODS

Reagents and Enzymes. Reagents were obtained as described (3) with the following additions: Calf thymus DNA was obtained from Sigma; Mono Q resin, poly(dA) (average length, 400 nucleotides), and $(dT)_{18}$ [oligo(dT)] were obtained from Pharmacia.

Extracts. The preparation of crude HeLa cytoplasmic extracts, cellular fractions (CF) (I′, II, and IIA), and purified proteins, PCNA, polymerase α-primase complex, and T antigen, has been described (3, 6, 7).

Anion-Exchange Chromatography. All solutions contained buffer A [30 mM Hepes (from 1 M Hepes stock solution at pH 7.8 at room temperature)/0.25% inositol/0.25 mM EDTA/10% (vol/vol) glycerol/1 mM dithiothreitol]. A 10-ml Mono Q column was equilibrated with 100 ml of buffer A containing 25 mM KCl. Approximately 100 mg of CF IIA was loaded onto the column. The column was then washed with 30 ml of buffer A containing 25 mM KCl, and material was eluted with a 36-ml linear gradient from 25 to 250 mM KCl, held at 250 mM KCl for 10 ml, and then eluted with a 36-ml linear

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Abbreviations: RP, replication protein; BuPh-dGTP, [N^2-*p-n*-butylphenyl)-9-(2-deoxy-β-D-ribofuranosyl)guanine 5′-triphosphate]; PCNA, proliferating-cell nuclear antigen; CF, cellular fraction; SV40, simian virus 40; T antigen, tumor antigen.

gradient from 250 to 1000 mM KCl. Fractions (2 ml) were collected and assayed for conductivity, protein content, and DNA polymerase activity. Aliquots were dialyzed against buffer A (0 mM KCl) until the conductivity was equivalent to 25 mM KCl and then assayed for replication activity.

Sucrose Gradient Sedimentation. Gradients were formed from 8 to 20% (wt/vol) sucrose in buffer A containing 250 mM KCl and allowed to stabilize for at least 1 hr at 4°C. Samples (200 μl) were centrifuged in an SW 50.1 rotor (Beckman) at 150,000 × g_{av} for 12.5 hr at 4°C. Fractions (250 μl) were collected from the bottom of the centrifuge tubes, dialyzed against buffer A until the conductivity was equivalent to 25 mM KCl, and assayed for DNA polymerase and replication activity.

Polymerase Assays. Assays were performed on two templates. Assays using activated calf thymus DNA contained, in a volume of 25 μl, 40 mM Tris·HCl (pH 6.5), 6 mM $MgCl_2$, bovine serum albumin (40 μg/ml), 5 μg of activated calf thymus DNA, 100 μM dATP, 100 μM dCTP, 100 μM dGTP, 50 μM [^{3}H]dTTP (70 mCi/mmol; 1 Ci = 37 GBq), and 1–5 μl of the fraction to be assayed. Assays using poly(dA)·oligo(dT) contained, in a volume of 25 μl, 40 mM Tris·HCl (pH 6.5), 6 mM $MgCl_2$, bovine serum albumin (40 μg/ml), 10% glycerol, 1 μg of poly(dA)·oligo(dT) (1:1 molar ratio) (15), and 1–5 μl of the fraction to be assayed. Assays using poly(dA)·oligo(dT) were performed in the presence or absence of 40 ng of PCNA per reaction mixture. All reaction mixtures were assembled on ice and then placed at 37°C. One unit was defined as the incorporation of 1 nmol of dNTP in 30 min at 37°C.

Preparation of SJK132 Antibody. Neutralizing polymerase α-specific monoclonal antibody SJK132 (18) was obtained from the American Type Culture Collection, purified by chromatography on protein A-Sepharose as described (18), and dialyzed against 5 mM Tris·HCl, pH 7.5/50 mM NaCl. To measure antibody inhibition, reaction mixtures were assembled as described for DNA polymerase assays using poly(dA)·oligo(dT) (plus 40 ng of PCNA) with the exception that dNTPs were omitted from the reaction mixture. Equal units of sucrose gradient-purified polymerase activity (fraction 12) or polymerase α–primase were added to the reaction mixture followed by SJK132 antibody or buffer. The reaction mixture was incubated on ice for 30 min and then dNTPs were added and incubation proceeded at 37°C for 30 min. No inhibition by buffer was seen at levels that resulted in 90% inhibition of polymerase α–primase activity on the synthetic template.

Inhibition Studies with [N^2-(*p-n*-butylphenyl)-9-(2-deoxy-β-D-ribofuranosyl)guanine 5′-triphosphate] (BuPh-dGTP). BuPh-dGTP was kindly provided by George Wright (University of Massachusetts Medical School, Worcester). Reaction mixtures were assembled as described for DNA polymerase assays using poly(dA)·oligo(dT) (plus 40 ng of PCNA). Sucrose fraction 12 activity (0.1–0.2 unit) or 0.3–0.4 unit of polymerase α–primase activity were added with mixing followed by 1 μl of the appropriate dilution of BuPh-dGTP. Incubation proceeded at 37°C for 10 min. Serial dilutions (1:10) of BuPh-dGTP were tested and the data from two experiments were in good agreement with each other.

DNA Replication Assays. Replication assays were performed as described (3). Reconstitution assays contained, in 25 μl, 80 ng of SV40 origin-containing plasmid pUC.HSO, 28 μg of CF I′ [containing replication protein A (RP-A), PCNA, and replication protein C (RP-C)], 1 μg of T antigen, 2.5 units of affinity-purified polymerase α–primase, and 5 μl of fractions to be assayed. Reaction mixtures were assembled on ice and then incubated at 37°C for 2 hr. Products of the reaction were quantitated for acid-insoluble radioactivity or separated by agarose gel electrophoresis and visualized by autoradiography. Background activity, obtained in the absence of an SV40 origin of DNA replication, was subtracted.

RESULTS

Fractionation of Cell Extracts and Reconstitution of SV40 DNA Replication. We have reported (3) the resolution of the crude HeLa cell cytoplasmic extract into two fractions, CF I′ and CF II, both of which are absolutely required for the replication of DNA molecules containing the SV40 origin. Further fractionation of CF I′ yielded PCNA, RP-A, and RP-C, whose roles in the SV40 DNA replication reaction have been described (6–8, 10). CF II contained all of the detectable DNA polymerase activity present in the fractionated cell extract. We purified DNA polymerase α–primase complex to near homogeneity from CF II by immunoaffinity chromatography. However, we found that the purified polymerase α–primase was not capable of reconstituting efficient SV40 DNA replication in the presence of CF I′ and T antigen. The flow-through fraction from the polymerase α–primase immunoaffinity column, designated CF IIA, was also required (3). To identify the essential component(s) present within CF IIA, this fraction was subjected to anion-exchange chromatography on Mono Q resin. Fig. 1*A* shows a typical profile obtained by elution of the Mono Q column with a

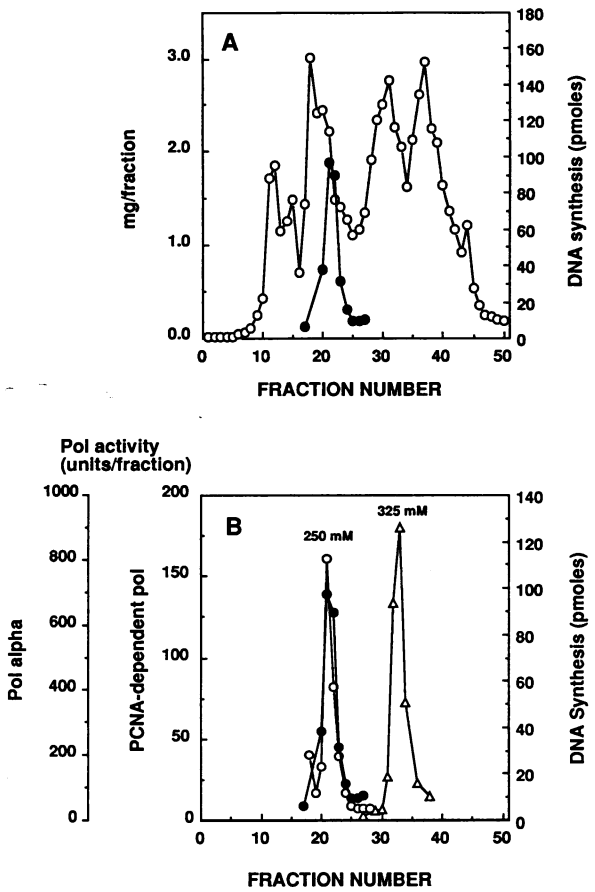

FIG. 1. Ion-exhange chromatography of cellular fraction CF IIA. CF IIA was fractionated by column chromatography on Mono Q resin and fractions were assayed for DNA polymerase and DNA replication activities. (*A*) Elution profile showing protein content in mg per fraction (○) and DNA replication activity in pmol (●) as assayed in the reconstituted SV40 DNA replication system. (*B*) Elution profile showing replication activity (●) assayed as above and DNA polymerase activity in total units per fraction assayed with either activated DNA (△) or poly(dA)·oligo(dT) (○) as template. Assays with the latter template were carried out in the presence of 40 ng PCNA. For clarity of presentation, only the activities in the regions of the peaks are shown. The activities were near background levels elsewhere. The concentration of KCl required to elute each peak is indicated. Pol alpha, DNA polymerase α.

gradient of KCl from 50 to 1000 mM. Fractions were assayed for their ability to support DNA replication in reaction mixtures containing T antigen, CF I', and affinity-purified DNA polymerase α–primase complex. We reproducibly observed a single peak of DNA replication activity eluting from the column at a salt concentration of 250 mM. In control experiments we verified that the observed DNA replication was absolutely dependent upon T antigen and the presence of a wild-type SV40 origin in the template. Replication was also dependent upon DNA polymerase α–primase and CF I' (data not shown). Analysis of the column fractions also revealed the presence of a nuclease activity (eluting at a slightly lower salt concentration) that stimulated repair synthesis, which was not dependent on T antigen or the viral replication origin. Fractions giving rise to this nonreplicative synthesis were not studied further.

Replication Activity Cofractionates with a PCNA-Dependent DNA Polymerase Activity. As mentioned above, the progenitor of fraction CF IIA contained all of the detectable DNA polymerase activity present in our crude cellular extracts. Therefore, it was of interest to determine whether any of the fractions eluted from the Mono Q column contained DNA polymerase activity. The column fractions were assayed for DNA polymerase activity with either activated calf thymus DNA or poly(dA)·oligo(dT) as template. Since PCNA has been shown to stimulate calf thymus DNA polymerase δ in the presence of poly(dA)·oligo(dT), it was included in all assays with this template. Two distinct peaks of DNA polymerase activity were observed. One peak of activity, which was detectable with either activated calf thymus DNA or poly(dA)·oligo(dT), was eluted at 325 mM KCl (Fig. 1*B*). This activity was not stimulated by PCNA on either template and was sensitive to inhibition by aphidicolin and monoclonal antibodies against DNA polymerase α (data not shown). We conclude that this peak represents a small amount of residual DNA polymerase α–primase not removed during the immunoaffinity chromatography step. The 325 mM KCl peak did not stimulate DNA replication in the reconstitution assay (see Fig. 1*A*) since the reaction mixtures contained saturating amounts of affinity-purified DNA polymerase α–primase.

A second peak of DNA polymerase activity was detected with the synthetic template poly(dA)·oligo(dT), eluting at 250 mM KCl. This activity was almost completely dependent upon the presence of PCNA, as only a small and rather

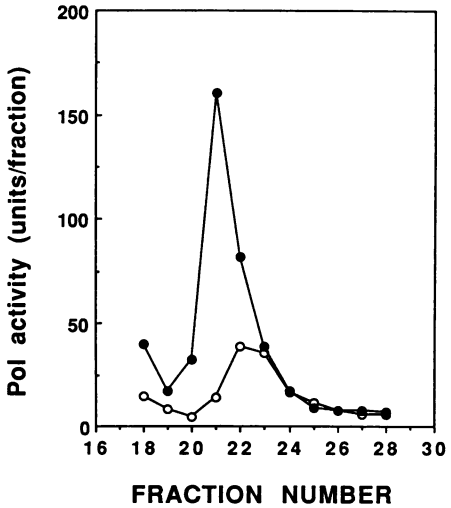

FIG. 2. PCNA-dependence of the replication-associated DNA polymerase activity. The DNA polymerase (Pol) that eluted at 250 mM KCl from the Mono Q column (Fig. 1) was assayed on the primer template poly(dA)·oligo(dT) in the presence (●) or absence (○) of 40 ng of PCNA.

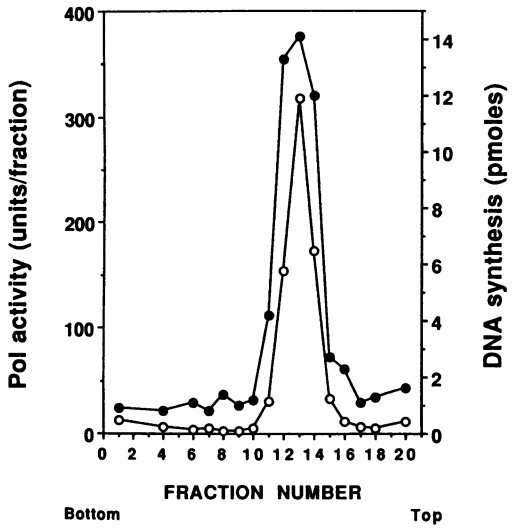

FIG. 3. Cosedimentation of the PCNA-dependent DNA polymerase with DNA replication activity in the reconstituted system. Fractions corresponding to the peak of DNA replication activity from the Mono Q column (fractions 21 and 22, Fig. 1*B*) were pooled and concentrated by batch elution from a 1-ml Mono Q column. The protein was then fractionated by sedimentation in a 8–20% sucrose gradient. The profile shows DNA polymerase activity (Pol; total units per fraction) determined by assays on poly(dA)·oligo(dT) in the presence of 40 ng of PCNA per reaction mixture (○) or SV40 DNA replication activity in the reconstituted system (●).

variable amount of DNA polymerase activity was observed in the absence of PCNA (Fig. 2). This polymerase had low activity with activated calf thymus DNA and was stimulated about 2-fold by the further addition of PCNA (data not shown). As illustrated in Fig. 1*B*, the peak of PCNA-dependent DNA polymerase activity coincided precisely with the peak of SV40 DNA replication activity detected by the reconstitution assay. These data strongly suggested a requirement for a second DNA polymerase in the SV40 cell-free system.

Replication Activity and PCNA-Dependent DNA Polymerase Activity Cosediment. To assess further the association of the PCNA-dependent DNA polymerase with DNA replication, the fractions containing peak replication activity from the Mono Q column (fractions 21 and 22) were pooled and subjected to velocity sedimentation in an 8–20% sucrose gradient. Fractions from the gradient were assayed for DNA polymerase activity with poly(dA)·oligo(dT) in the presence of PCNA and for DNA replication activity in the reconstituted SV40 system. Fig. 3 shows the activity profile from one such gradient. We observed a single peak of PCNA-dependent DNA polymerase activity with a sedimentation coefficient of about 4 S. This peak cosedimented with an activity required to reconstitute efficient SV40 DNA replication *in vitro*. As shown in Table 1, the observed DNA replication required DNA polymerase α–primase and the

Table 1. Requirements for reconstitution of SV40 DNA replication *in vitro*

Component omitted	DNA synthesis, pmol
None*	13.2
T antigen	2.0
SV40 origin	1.3
DNA polymerase α	2.2
PCNA-dependent DNA polymerase	1.9

*Components of the complete reaction mixture are listed in Fig. 4.

PCNA-dependent DNA polymerase activity and was also dependent upon T antigen and the SV40 origin.

The products synthesized in reconstituted reaction mixtures containing both DNA polymerase activities were analyzed by agarose gel electrophoresis (Fig. 4). The distribution of products was similar to that observed with the crude extract (1) although fewer monomeric circles and more high molecular weight material was observed. The reconstituted reaction was also significantly less efficient than that observed with crude extract. It seems likely that the reconstituted reaction lacks stimulatory factors that are present in less pure fractions. When the PCNA-dependent DNA polymerase activity was omitted from the reaction, the total synthesis was greatly reduced (Fig. 4 and Table 1). Alkaline gel electrophoresis of the residual products synthesized under these conditions revealed that they consisted primarily of short nascent chains less than 600 nucleotides long (data not shown).

Characteristics of the PCNA-Dependent DNA Polymerase Activity. The biochemical characteristics of the PCNA-dependent DNA polymerase activity purified through the sucrose gradient step are summarized in Table 2. Unlike DNA polymerase α–primase complex, this DNA polymerase contained no detectable primase activity and was insensitive to inhibition by SJK132, a neutralizing monoclonal antibody directed against DNA polymerase α. Like DNA polymerase α–primase, the PCNA-dependent polymerase was sensitive to the inhibitor aphidicolin. Both DNA polymerase α and DNA polymerase δ are sensitive to aphidicolin (15, 19, 20). However, the two polymerases can be distinguished by a difference in sensitivity to the nucleotide analog BuPh-dGTP, which is a relatively selective inhibitor of DNA polymerase α (21–23). We found that our purified human DNA polymerase α was inhibited 50% by 5μM BuPh-dGTP, whereas a 30-fold greater concentration was required to achieve 50% inhibition of the PCNA-dependent DNA polymerase activity. Thus, these biochemical characteristics, and in particular the requirement for PCNA and the insensitivity to BuPh-dGTP, are consistent with the hypothesis that the PCNA-dependent activity is, in fact, human DNA polymerase δ.

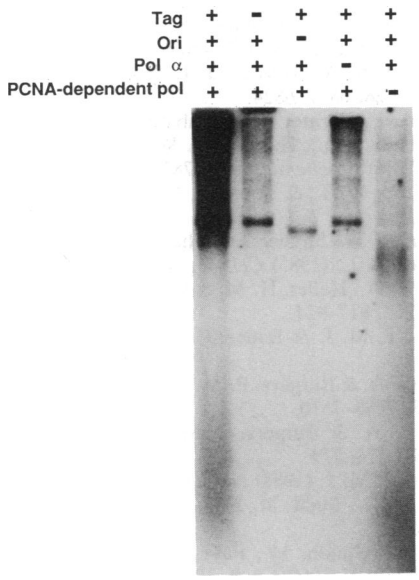

FIG. 4. Agarose gel electrophoresis of the DNA products synthesized in the presence of the two DNA polymerases. The complete reconstituted SV40 replication reaction mixture (25 μl) contained 1 μg of T antigen (Tag), 24 μg of CF I′, 2.5 units of affinity-purified DNA polymerase α–primase complex (pol α), 0.5 unit of PCNA-dependent DNA polymerase (sucrose gradient fraction 12), and 80 ng of the plasmid pUC.HSO, which contains the SV40 replication origin (Ori). The individual components were omitted from the reaction mixture as indicated (−). For the lane marked Ori −, the plasmid pUC9, which lacks the SV40 origin, was substituted for pUC.HSO +, Component present.

Table 2. Biochemical characteristics of DNA polymerases required for SV40 DNA replication *in vitro*

	Polymerase	
	PCNA-dependent	α–primase
Primase activity	No	Yes
Stimulation by PCNA	Yes	No
IC_{50} by BuPh-dGTP, μM	150	5
Inhibition by SJK132, %	10	99
Required for SV40 DNA replication *in vitro*	Yes	Yes

DISCUSSION

We have been systematically fractionating HeLa cell extracts to define the cellular proteins that are essential for the cell-free synthesis of SV40 DNA. The results to date indicate that the replication machinery is quite complex, as a minimum of seven cellular components are required to reconstitute efficient DNA synthesis *in vitro* (3–9). In this report we have described the further resolution of one of these components, CF II. This fraction contains a mixture of proteins, including all of the detectable DNA polymerase activity in the original crude cytoplasmic extract. By a series of fractionation steps, CF II has been separated into two components. One of these components is the DNA polymerase α–primase complex, which was obtained in essentially homogenous form by immunoaffinity chromatography (3). The second component is a PCNA-dependent DNA polymerase, distinct from DNA polymerase α. We have shown that this second DNA polymerase copurifies with SV40 DNA replication activity through ion-exchange chromatography and sucrose gradient sedimentation. Although the DNA polymerase is not yet homogeneous, our data provide strong evidence that two cellular DNA polymerases are required to reconstitute SV40 DNA replication *in vitro*.

It seems likely that the PCNA-dependent DNA polymerase that has been identified in extracts of HeLa cells is the human analog of DNA polymerase δ, originally purified from calf thymus (for review, see refs. 12 and 20). A similar enzyme was subsequently purified from human placenta (24). DNA polymerase δ is chromatographically and immunologically distinct from DNA polymerase α. In addition, it is less sensitive than DNA polymerase α to the inhibitory nucleotide analog BuPh-dGTP. Perhaps the most specific defining characteristic of DNA polymerase δ is its strong dependence on PCNA when templates containing low primer-to-template ratios are used. All of these properties of DNA polymerase δ are shared by the activity that we have implicated in SV40 DNA replication *in vitro*. We have not yet verified the presence of the exonuclease activity reported to be associated with DNA polymerase δ due to the presence of low levels of contaminating nuclease activity in our most purified preparations. Further purification of the enzyme has proven challenging since the enzyme activity is unstable after the anion-exchange chromatography step.

We have tested the ability of highly purified calf thymus DNA polymerase δ [kindly provided by K. Downey and A. So (Univ. of Miami Medical School)] to replace CF IIA in our reconstituted SV40 DNA replication system. In these experiments the purified calf thymus enzyme could not substitute for the HeLa fraction. This may reflect a species-specific interaction between DNA polymerase δ and other components of the replication system. Such an interaction has been

invoked to explain the observation that calf thymus DNA polymerase α–primase complex will not substitute for the human enzyme in the SV40 cell-free system (4). Alternatively, the inability of the calf thymus DNA polymerase δ to support SV40 DNA replication *in vitro* may reflect the inactivation of a critical activity or the loss of an accessory factor(s) during extensive purification.

Genetic and biochemical studies of the budding yeast *Saccharomyces cerevisiae* lend further support to the hypothesis that two DNA polymerases are required for DNA replication in eukaryotes. Yeast DNA polymerase I is analogous to mammalian DNA polymerase α–primase and has been shown to be essential for DNA replication (25). Yeast DNA polymerase III has biochemical properties similar to those of mammalian DNA polymerase δ, including sensitivity to inhibition by aphidicolin, relative resistance to inhibition by BuPh-dGTP, and association with an exonuclease activity (26, 27). Perhaps most striking is the finding that yeast DNA polymerase III is stimulated by mammalian PCNA or by a 28-kDa yeast analog of mammalian PCNA, designated yPCNA (28–30). DNA polymerase III is encoded by the *S. cerevisiae* cell cycle division gene, *CDC2* (31, 32). Since *cdc2* mutants are not viable at the nonpermissive temperature and arrest in S phase, it seems likely that DNA polymerase III, like DNA polymerase I, is involved in the replication of yeast chromosomal DNA.

The possible involvement of DNA polymerase δ in SV40 DNA replication was originally inferred from the observation that PCNA is required for efficient chain elongation in a reconstituted SV40 DNA replication system (3, 10). In the absence of PCNA, DNA synthesis is greatly reduced, and the synthetic products consist of short nascent chains that originate preferentially, but not exclusively, from the region of the viral replication origin (16). Analysis of the products that originate outside of the immediate origin region indicates that they are synthesized primarily on the lagging-strand template. We have obtained similar results with a system containing only the purified proteins T antigen, RP-A, RP-C, and DNA polymerase α–primase complex (unpublished results). This purified system, which lacks DNA polymerase δ activity, is capable of initiating the synthesis of short nascent strands on duplex DNA molecules containing the SV40 origin. In the immediate vicinity of the origin the products are synthesized from both template strands at about equal frequency, whereas distal to the origin the products are synthesized from the lagging-strand template preferentially. Based on the observed asymmetry of the nascent strands synthesized in the absence of PCNA, it has been suggested that DNA polymerase α mediates lagging-strand synthesis at the replication fork, and DNA polymerase δ mediates leading-strand synthesis (16, 17). Although there is as yet no direct evidence for this model, it is consistent with the known biochemical properties of the two polymerases (i.e., DNA polymerase α has an associated primase activity and is not very processive, and DNA polymerase δ lacks primase activity and is highly processive) (15). It should now be possible to use the reconstituted SV40 DNA replication system to address more directly the functional role of DNA polymerase δ in DNA replication.

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