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A 21S Enzyme Complex from HeLa Cells That Functions in Simian Virus 40 DNA Replication in Vitro†

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Received October 30, 1989; Revised Manuscript Received March 9, 1990

ABSTRACT: A sedimentable complex of enzymes for DNA synthesis was partially purified from the combined low-salt nuclear extract–postmicrosomal supernatant solution of HeLa cell homogenates by poly(ethylene glycol) precipitation in the presence of 2 M KCl, discontinuous gradient centrifugation, Q-Sepharose chromatography, and velocity gradient centrifugation. In addition to the previously described 640-kDa multiprotein DNA polymerase α–primase complex [Vishwanatha et al. (1986) *J. Biol. Chem. 261*, 6619-6628], the enzyme complex also has associated topoisomerase I, DNA-dependent ATPase, RNase H, DNA ligase, a simian virus 40 origin recognition, dA/dT sequence binding protein [Malkas & Baril (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 70-74], and proliferating cell nuclear antigen. Essentially all of the T antigen dependent simian virus 40 in vitro replication activity in the combined nuclear extract–postmicrosomal supernatant solution resides with the sedimentable complex of enzymes for DNA synthesis. Sedimentation analysis on a 10–35% glycerol gradient in the presence of 0.5 M KCl indicates that the enzyme complex is 21S. The associated enzymes for DNA synthesis and in vitro simian virus 40 replication activity cofractionate throughout the purification of the 21S complex. The DNA polymerase and in vitro simian virus 40 replication activities are both inhibited by monoclonal antibody (SJK 132-20) to human DNA polymerase α and by 5–10 μM butylphenyl-dGTP, indicating that the association of DNA polymerase α with the 21S enzyme complex is essential for the initiation of SV40 DNA replication in vitro.

Chromosomal DNA replication in animal cells is a complex process, the mechanism and regulatory control of which are poorly understood. An understanding of this process will require information on the organization and control of the DNA synthesizing machinery, as well as replicons, replication origins (ori),[1] and their cis-acting control elements. Through the development of improved purification procedures, it is now apparent that DNA polymerase α, and the analogous DNA polymerase I from yeast (Campbell, 1986), as isolated from a broad variety of eukaryotes exists as a multiprotein complex that includes primase and other proteins in addition to the polymerase α catalytic subunit [reviewed in Kaguni and Lehman (1988) and Lehman and Kaguni (1989)]. The results of studies of the DNA synthesizing machinery in prokaryotes, by the use of in vitro systems for replication of bacteriophage DNAs (Kornberg, 1980, 1982, 1988; Alberts, 1985; Richardson, 1983), however, have demonstrated that DNA replication requires the concerted action of several enzymes and nonenzymic proteins in addition to the DNA polymerase holoenzymes. This was further established by recent studies on the initiation of replication of *Escherichia coli* (Funnell et al., 1986; Kornberg, 1988) and λ bacteriophage (Mensa-Wilmot et al., 1989) chromosomes in vitro using purified proteins.

Simian virus 40 (SV40)[1] replication uses the host cell DNA synthesizing apparatus, and the only viral-encoded protein that

† This work was supported by U.S. Public Health Service Grants P-3012708 and CA-15187 and by National Research Service Award CA-08173 to L.H.M.
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[1] Abbreviations: AAN, aminoacetonitrile hemisulfate; bp, base pair(s); BSA, bovine serum albumin; BuPdGTP, N^2-(*p-n*-butylphenyl)-dGTP; dA/dT, runs of deoxyadenines or deoxythymines; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA-Na_3, ethylenediaminetetraacetic acid trisodum salt, pH 7.5; EGTA-Na_3, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid trisodium salt, pH 7.5; ori, replication origin; PCNA, proliferating cell nuclear antigen; PEG, poly(ethylene glycol); NE, 0.15 N KCl nuclear extract; S-3, postmicrosomal supernatant solution; P-4, sedimentable subfraction of the combined nuclear extract–postmicrosomal supernatant solution; S-4, nonsedimentable subfraction of the combined nuclear extract–postmicrosomal supernatant solution; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; ss, single stranded; SV40, simian virus 40; T-ag, simian virus 40 large, tumor (T) antigen; Tris, tris(hydroxymethyl)aminomethane; TBS, 50 mM Tris-HCl, pH 7.5, and 0.05 M NaCl; TCA, trichloroacetic acid.

0006-2960/90/0429-6362$02.50/0

is required is large T antigen (T-ag).[1] The advent of an in vitro system for T-ag-dependent, ori-specific initiation of SV40 DNA replication provides a means for the analysis of the enzymological machinery for chromosomal DNA replication in permissive cells (Li & Kelly, 1984; Stillman & Gluzman, 1985; Wobbe et al., 1985; Yamaguchi & DePamphilis, 1986). The properties of this system and its attributes for the analysis of the DNA synthesizing machinery in animal cells have been the subject of recent reviews (Kelly, 1988; Kelly et al., 1988; Stillman, 1989).

An association of the 640-kDa multiprotein DNA polymerase α–primase complex (Vishwanatha et al., 1986a) from HeLa cells with other enzymes for DNA synthesis such as DNA-dependent ATPase, DNA ligase, topoisomerase I, etc. to form a sedimentable complex was observed previously (Baril et al., 1988; Hickey et al., 1988). In this paper, we report on the isolation and partial characterization of the properties of this enzyme complex and present evidence that the complex is essential for origin-specific and T-ag-dependent initiation of SV40 replication in vitro.

Materials and Methods

Materials

Radioactively labeled nucleotides were obtained from Amersham or Dupont–NEN. Unlabeled nucleotides, oligodeoxynucleotides, and Q-Sepharose were purchased from Pharmacia Biotechnology Inc. N^2-(*p-n*-Butylphenyl)-dGTP (BuPdGTP)[1] (Khan et al., 1984) was a gift from Dr. George Wright, University of Massachusetts Medical School. Aminoacetonitrile hemisulfate (AAN)[1] was obtained from Pfaltz-Bauer Inc. Poly(dA)$_{1000}$, poly(dC)$_{1000}$, and poly(dT)$_{1000}$ were obtained from Midland Certified Reagents. Pancreatic DNase-activated DNA was prepared by a published procedure (Baril et al., 1977). The PSVO$^+$ plasmid, containing the *Hin*dIII–*Sph*I SV40 ori DNA fragment cloned into pAT153 DNA (Li et al., 1986), and plasmids containing deletions in the SV40 ori sequence were obtained by a published procedure (Malkas & Baril, 1989). SV40 T antigen was obtained from extracts of HeLa cells that were infected with the recombinant adenovirus AdSVR284 and purified by immunoaffinity chromatography on a column of agarose-linked anti-T antigen antibody PAb 419 (Simanis & Lane, 1985). Restriction endonucleases *Dpn*I and *Sal*I were obtained from New England Biolabs and Bethesda Research Laboratories, respectively. HeLa S_3 cells were grown as previously described (Chiu & Baril, 1975). The hybridoma cell line (1640) secreting antibody SJK 132-20 to human DNA polymerase α (Tanaka et al., 1982) was obtained from the American Type Culture Collection. The antibody (IgG fraction) was purified from mouse ascites fluid by chromatography on a protein A linked agarose column purchased from Repligen Inc. Affinity-purified IgG for 19F4 mouse monoclonal antibody (Ogata et al., 1987) to proliferating cell nuclear antigen (PCNA)[1] was purchased from American Biotech Inc. All other reagents used were described in previous publications from this laboratory.

Methods

Subcellular Fractionation. The preparation and subfractionation of HeLa cell homogenates were by modification of a published procedure (Vishwanatha et al., 1986a). A 30% homogenate of exponentially growing HeLa cells (usually 25–50 g wet weight) was prepared and fractionated into the low-salt (0.15 M KCl) extract of isolated nuclei (NE)[1] and postmicrosomal supernatant solution (S-3).[1] The combined NE/S-3 fraction was adjusted to 2 M KCl and 5% poly(ethylene glycol) 8000 concentrations followed by rocking at 4 °C for 60 min and centrifugation at 16000*g* for 30 min. The resulting supernatant solution was dialyzed against 50 mM Tris-HCl, pH 7.5, 0.15 M KCl, 1 mM DTT,[1] 1 mM EDTA-Na_3,[1] 1 mM EGTA-Na_3,[1] 1 mM PMSF,[1] and 1 mM AAN (buffer A) containing 0.25 M sucrose. The dialyzed fraction was clarified by centrifugation at 13000*g* for 30 min; the supernatant solution was layered onto a 2 M sucrose cusion in buffer A and recentrifuged at 100000*g* for 16 h at 4 °C. Following centrifugation, the supernatant solution (S-4 fraction)[1] and the 2 M sucrose interphase (P-4 fraction)[1] were successively removed by aspiration. The P-4 and S-4 fractions were dialyzed against 50 mM Tris-HCl, pH 7.5, 0.15 M KCl, 1 mM EDTA-Na_3, and 10% glycerol (buffer B) and were used immediately or stored in aliquots at −80 °C.

Q-Sepharose Chromatography. The P-4 or S-4 fraction was loaded onto a column of Q-Sepharose (1 cm^3 bed volume/25 mg of protein) equilibrated with buffer B. After being washed with 8 column volumes of buffer B, the column was eluted with 10 column volumes of a continuous gradient of increasing KCl concentration from 0.05 to 0.5 M in 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA-Na_3, and 10% glycerol (TDEG buffer). Fractions of 0.5 mL were collected and assayed for protein and the respective enzyme activities. The concentration of KCl throughout the gradient was determined by conductivity measurements. Fractions containing the peak of the eluted enzyme activities were pooled, dialyzed against 0.15 M KCl in TDEG buffer, and used immediately or stored at −80 °C. The individual enzyme activities were stable to storage under these conditions for at least 6 months but were sensitive to repeated freeze–thawings.

Zonal Glycerol Gradient Centrifugation. A 10–35% glycerol gradient in buffer B containing 0.5 M KCl was formed over a 0.5-mL cushion of 2 M sucrose in polyallomer tubes for the SW50.1 Spinco rotor. A 0.1-mL aliquot (0.2 mg of protein) of the P-4 fraction was loaded onto the preformed gradient and centrifuged at 150000*g* for 16 h at 4 °C. After centrifugation, the gradient was fractionated by tube puncture from the bottom; 0.1-mL fractions were collected and assayed for enzyme activities as described for the results from individual experiments. Sedimentation of marker proteins (porcine thyroglobulin, 19 S; horse spleen apoferritin, 17 S; and yeast alcohol dehydrogenase, 7 S) run on parallel gradients indicated that the 16-h gradient was isokinetic. Sedimentation values were calculated according to the procedure of Siegel and Monty (1966).

Enzyme Assays. DNA polymerase α activity with activated and primed, single-stranded (ss)[1] DNA templates was assayed according to published procedures (Lamothe et al., 1981; Vishwanatha et al., 1986b). One unit of DNA polymerase α activity equals 1 nmol of total dNMP incorporated into DNA per hour at 35 °C. DNA primase activity was assayed by measuring the incorporation of [^{3}H]GTP (800–1000 cpm/pmol) into acid-insoluble material in the presence of poly(dC) as template (Vishwanatha & Baril, 1986). One unit of DNA primase activity equals 1 nmol of NMP incorporated into acid-insoluble material per hour at 30 °C. DNA polymerase α associated 3′ to 5′ exonuclease activity was assayed according to the procedure of Skarnes et al. (1986). DNA-dependent ATPase activity was assayed by measuring the conversion of [γ-^{32}P]ATP (2000 cpm/pmol) to the charcoal-nonadsorbable form in the presence of heat-denatured calf thymus DNA or poly(dT)$_{500}$ as previously described (Baril et al., 1988). One unit of ATPase activity equals the hydrolysis of 1 nmol of ATP per hour at 30 °C. DNA ligase activity was assayed according to a published procedure (Hickey et

al., 1988). One unit of DNA ligase activity is equal to 10000 cpm of 5′-[^{32}P]oligodeoxynucleotide rendered alkaline phosphatase resistant per hour at 30 °C. RNase H activity was assayed by measuring the conversion of [^{3}H]poly(A) (800 cpm/pmol) to acid-soluble material using [^{3}H]poly(A)·poly(dT) as substrate as previously described (Baril) et al., 1988). The specificity of hydrolysis of poly(dT)-hybridized [^{3}H]-poly(A) was assessed by running parallel assays using [^{3}H]-poly(A), [^{3}H]poly(dA), and [^{3}H]poly(dA)·poly(dT) as substrates. One unit of RNase H activity equals 1 nmol of [^{3}H]poly(A) converted to acid-soluble form per hour at 30 °C. Type I topoisomerase activity was assayed for the relaxation of pUC8 DNA according to the procedure of Liu and Miller (1981). Type II topoisomerase activity was assayed for the unknotting of *Crithidia fasciculata* kinetoplast DNA using the procedure of Miller et al. (1981). The dA/dT sequence recognition protein was assayed according to a published procedure using a nitrocellulose filter binding assay (Malkas & Baril, 1989).

In Vitro SV40 Replication Assay. The in vitro replication of pSVO^{+} plasmid DNA containing an insert of sequences of the SV40 replication origin was assayed according to the procedure of Wold and Kelly (1988) with minor modifications. The reaction (25-μL volume) contained 30 mM Hepes, pH 7.2, 7 mM $MgCl_2$, 0.5 mM DTT, 5 μCi of [α-^{32}P]dATP, 50 μM dATP, 100 μM each of dTTP, dCTP, and dGTP, 200 μM each of CTP, UTP, and GTP, 4 mM ATP, 40 mM phosphocreatine, 100 μg of creatine phosphokinase, 30 ng of pSVO^{+} or deletion-containing plasmid DNAs, T antigen (0.2–0.3 μg), the optimal amount predetermined by titration assays, and the designated amount of the respective protein fractions as described with the individual experiments. Incubation was at 35 °C for 1–3 h. For standard assays, the reaction was terminated by addition of 100 μg of yeast RNA (carrier) and precipitation by the addition of trichloroacetic acid (TCA)[1] to 10% concentration. The precipitated product was collected by filtration on glass fiber filters, washed with 95% ethanol, and dried, and the radioactivity was determined by liquid scintillation counting. One unit of SV40 replication activity is equal to the incorporation of 50 pmol of dNMP into SV40 DNA per hour under the standard assay condition.

For analysis of the replication product, the DNA was isolated by phenol/chloroform extraction followed by precipitation with 2-propanol in the presence of 2 M ammonium acetate. The isolated DNA was resuspended in 10 mM Tris, pH 8.0, and 1 mM EDTA-Na_3 and divided into two aliquots. One aliquot was incubated with 8 units of *Dpn*I to digest the methylated input plasmid DNA and 20 units of *Sal*I to linearize (cut) the *Dpn*I-resistant in vitro replicated DNA (Li & Kelly, 1984) while the other aliquot (undigested, uncut) was left at 4 °C. Aliquots of the digested (cut) and undigested (uncut) product were electrophoresed on 1.5% agarose gels in TBE buffer (89 mM Tris–borate, 89 mM boric acid, and 1 mM EDTA-Na_3, pH 7.8). Gels were dried under vacuum and exposed to Kodak XAR-5 film at −80 °C. For quantitation, the gel bands of interest were excised from the gel after autoradiography, and radioactivity was determined by liquid scintillation counting.

Polyacrylamide Gel Electrophoretic Analysis of Proteins. Polyacrylamide gel electrophoretic analyses of proteins under nondenaturing and denaturing conditions were performed as previously described (Lamothe et al., 1981; Vishwanatha et al., 1986a). The protein-stained bands were developed by Coomassie blue or silver staining.

Immunoblot Analysis. Protein samples were electrophoresed under denaturing or nondenaturing conditions according to published procedures (Lamothe et al., 1981; Vishwanatha et al., 1986a). Electroblotting of the separated proteins was performed according to the procedure of Towbin and co-workers (Towbin et al., 1979) with modifications. After electrophoresis, the protein bands were electrotransfered at 20 V overnight at 4 °C onto nitrocellulose membranes that were presoaked in transfer buffer (24 mM Tris–base and 192 mM glycine, pH 8.3) containing 20% methanol, and for transfer from denatured gels, the buffer also contained 0.01% SDS.[1] Immunoblot analysis of the electrotransferred proteins was performed by modification of a published procedure (Johnson et al., 1984). After electrotransfer, the membranes were blotted dry, soaked for 1 h at room temperature in blocking buffer (20 mM Tris-HCl, pH 7.5, 40 mM NaCl, 0.1% NP-40, and 5% Carnation non-fat dry milk), washed 3 times for 5 min in TBS[1] (50 mM Tris-HCl, pH 7.5, and 0.05 M NaCl), and then incubated for 1–3 h at room temperature with primary antibody as described with the individual experiments. The membranes were washed 3 times for 5 min each in TBS to remove the unreacted primary antibody and incubated for 1 h with a 1:1000 dilution of alkaline phosphatase conjugated sheep anti-mouse IgG in 15 mM Tris-HCl, pH 7.5, 37.5 mM NaCl, 1.65% Carnation nonfat dry milk, 0.033% NP-40, and 3.3% NaN_3 followed by three 5-min washes in TBS. Color was developed by incubating in a diethanolamine buffer, pH 9.8, containing 4 mM $MgCl_2$, 0.005% 5-bromo-4-chloro-3-indolyl phosphate, and 0.01% nitroblue tetrazolium.

Other Methods. The neutralization of DNA polymerase α and SV40 in vitro replication activity by monoclonal antibody SJK 132-20 to human polymerase α (Tanaka et al., 1982) and inhibition by BuPdGTP were performed according to published procedures (Baril et al., 1988; Hickey et al., 1988). Protein was determined by the procedure of Bradford (1976) using bovine serum albumin (BSA)[1] as the standard.

For measuring the recovery of DNA and RNA in subfractions of NE/S-3, 500 mL of exponentially growing cultures of HeLa cells was metabolically labeled with either 0.2 μCi of [^{3}H]thymidine or 0.2 μCi of [^{3}H]uridine at 37 °C for 2 h. Cells were harvested and homogenates prepared and subfractionated as described under the section on the Subcellular Fractionation. An equal volume of ice-cold 20% TCA was added to aliquots of the untreated and PEG treated NE/S-3 and its subfractions, and after 15 min at 4 °C, the acid-insoluble material was collected on glass fiber filters by filtration. After the filters were dried, the radioactivity retained on the filters was measured by liquid scintillation counting.

Results

We previously showed that discontiuous gradient centrifugation of the combined low-salt nuclear extract–postmicrosomal supernatant fraction (NE/S-3) from HeLa cell homogenates separates a sedimentable (P-4) from a nonsedimentable (S-4) fraction of enzymes for DNA synthesis (Baril et al., 1988; Hickey et al., 1988). Most of the previously described 640-kDa DNA polymerase α–primase complex (Vishwanatha et al., 1986a), DNA-dependent ATPase, and RNase H activities in NE/S-3 sediment with the P-4 fraction while topoisomerase I and DNA ligase are recovered with both the P-4 and S-4 fractions (Hickey et al., 1988). A dA/dT sequence recognition protein that specifically binds to the 17 bp A/T-rich tract in the initiation zone of SV40 ori (Malkas & Baril, 1989) is also associated with the P-4 fraction. Essentially all of the T antigen dependent SV40 in vitro repli-

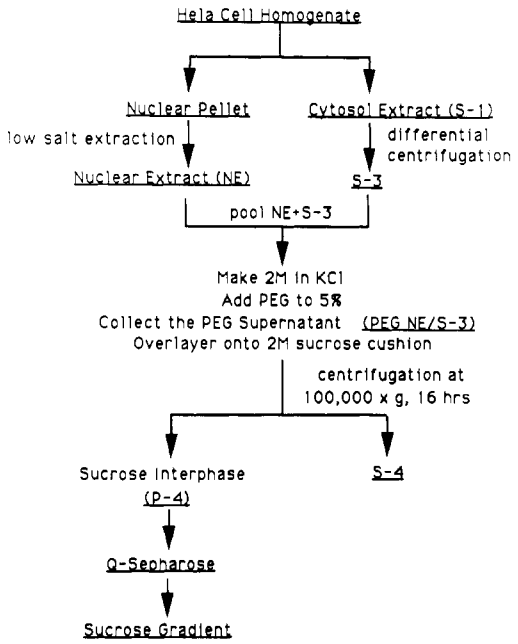

FIGURE 1: Flow diagram of the procedure used for purification of the 21S enzyme complex that functions in SV40 replication in vitro. Homogenates were routinely prepared from 20–50 g (wet weight) of HeLa cells. Details of the purification procedure are given under Materials and Methods.

cation activity is recovered with the sedimentable P-4 fraction that contains the complex of enzymes for DNA synthesis (Baril et al., 1988). In order to define its role in SV40 and cellular DNA replication, the sedimentable complex of enzymes for DNA synthesis has been partially purified and its properties further characterized.

Isolation of the 21S Enzyme Complex

Most of the SV40 replication activity in HeLa cell homogenates resides with cytoplasm while an additional 20% of higher specific activity is extracted from the isolated nuclei by 0.15 M KCl (Baril et al., 1988). The 0.15 M KCl nuclear extract does not stimulate the cytoplasmic SV40 replication activity. To increase the amount of starting material for isolation of the enzyme complex, however, the 0.15 M nuclear extract (NE) was combined with the cytoplasmic postmicrosomal supernatant solution (S-3). The sedimentable enzyme complex that is competent in T-ag-dependent replication of SV40 DNA was isolated and partially purified from the NE/S-3 fraction by the procedure outlined in Figure 1.

Poly(ethylene glycol) Precipitation of the NE/S-3 Fraction. The initial step in the isolation, PEG precipitation in the presence of 2 M KCl, removes about 50% of the protein (Figure 2A) and 70–80% of the nucleic acids (Figure 2B,C) present in the NE/S-3 fraction. However, more than 90% of the DNA polymerase α activity that functions with activated (gapped) and primed, ssDNA templates (Figure 2D), 80% of the primase activity (Figure 2E), and essentially all of the T-ag-dependent SV40 in vitro replication activity (Figure 2F) in the NE/S-3 fraction are recovered in the supernatant solution after centrifugation of the PEG precipitate. The addition of 5% PEG to NE/S-3 did not alter the SV40 replication activity in this fraction although higher concentrations of PEG (i.e., 10% or greater) inhibited the SV40 replication activity by approximately 60%.

Discontinuous Gradient Centrifugation. The distribution of the enzymes for DNA synthesis (i.e., 640-kDa polymerase α–primase complex, DNA-dependent ATPase, RNase H, topoisomerase I, and in vitro SV40 replication activities) in the P-4 and S-4 fractions of NE/S-3 remain the same after the PEG precipitation step (data not shown). In both cases, virtually all (i.e., more than 95%) of the T-ag-dependent SV40 in vitro replication activity is recovered with the P-4 fraction that contains the sedimentable complex of enzymes for DNA synthesis. Gel electrophoretic analysis shows that the products from reactions with P-4 obtained from untreated and PEG-treated NE/S-3 fractions are comparable and are very similar to those obtained for the synthesis with NE/S-3 from which

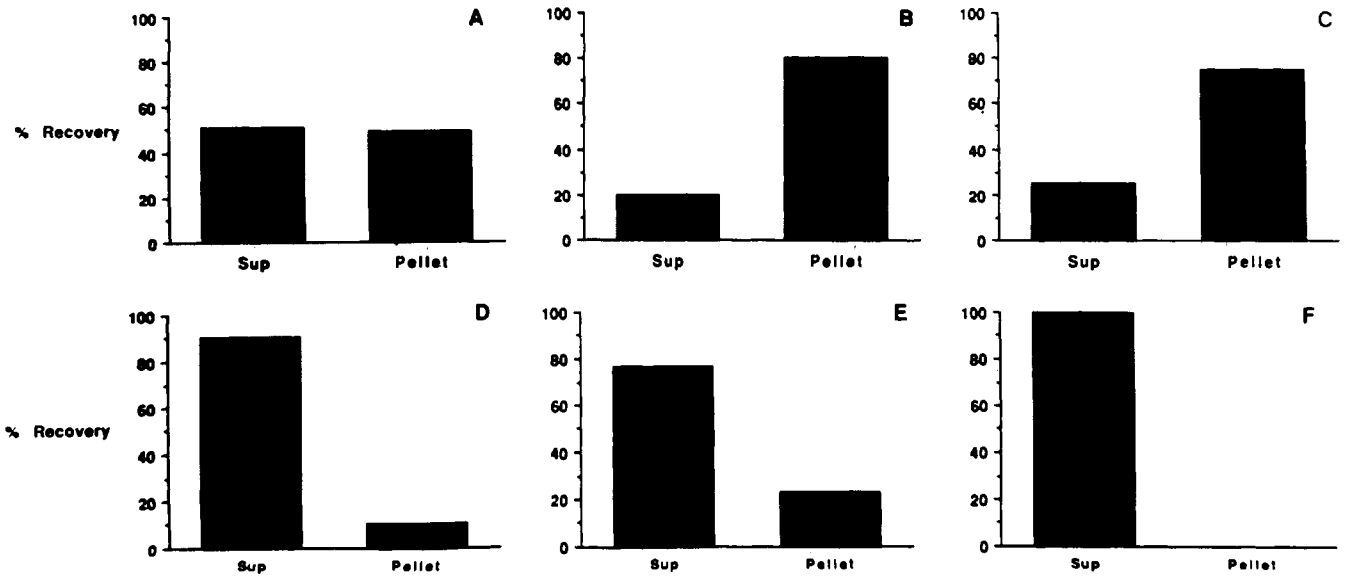

FIGURE 2: Recovery of protein, DNA, RNA, DNA polymerase, primase, and SV40 in vitro replication activity following PEG precipitation of the NE/S-3 fraction. The combined 0.15 M KCl nuclear extract–postmicrosomal supernatant fraction (NE/S-3) from a homogenate of 13 g of HeLa cells was treated with 5% (w/v) poly(ethylene glycol) 8000 (PEG) and the PEG-precipitated (pellet) material separated from the nonprecipitated supernatant solution (sup) by differential centrifugation as described under Materials and Methods. The percent recovery is relative to that present with the NE/S-3 fraction (100%). The recovery in the supernatant solution was measured directly after dialysis, and for the PEG-precipitated fraction, it was calculated by difference. (A) Protein, 100% value equals 190 mg. (B) DNA, 100% value equals 3.5×10^5 cpm of [^{3}H]DNA. (C) RNA, 100% value equals 9.5×10^6 cpm of [^{3}H]RNA. (D) DNA polymerase, 100% value equals 3800 units measured with activated DNA template. (E) Primase, 100% value equals 456 units. (F) SV40 in vitro replication activity, 100% value equals 498 units. The procedures for the assays and units of activity are described under Materials and Methods.

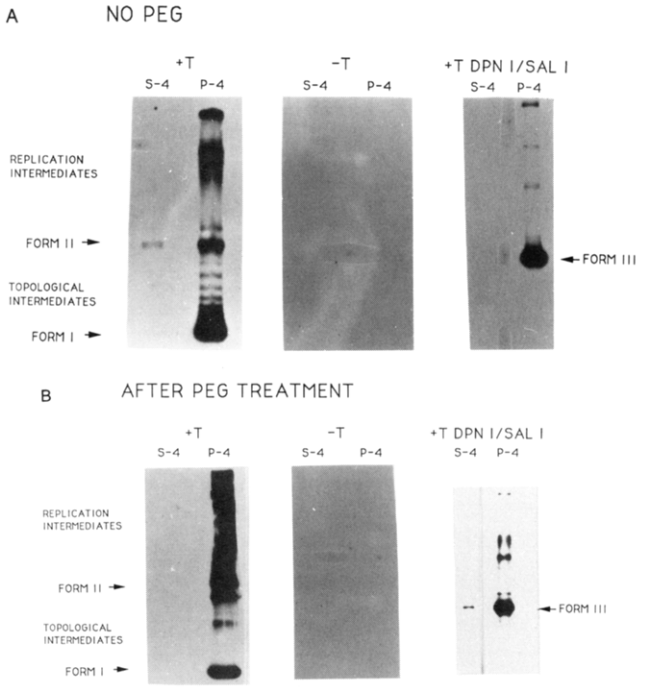

FIGURE 3: Gel electrophoretic analysis of the product from SV40 replication in vitro by the P-4 and S-4 subfractions of PEG-treated and nontreated NE/S-3. The P-4 and S-4 fractions were isolated directly from the NE/S-3 (no PEG treatment) or from the supernatant obtained following centrifugation of the 5% PEG-treated (PEG treated) NE/S-3 fraction. SV40 in vitro replication activity was assayed according to a modification of the procedure of Wold and Kelly (1988). The reactions containing P-4 from nontreated (32 μg of protein) or PEG-treated (28 μg of protein) NE/S-3 and S-4 from nontreated (22 μg of protein) or PEG-treated (19 μg of protein) NE/S-3 in the presence (+T) or absence (–T) of 0.3 μg of T antigen were incubated at 35 °C for 2 h; the DNA product was isolated by phenol/chloroform extraction, precipitated with 2-propanol, resuspended in 10 mM Tris-HCl, pH 8.0, and divided into two aliquots. One aliquot (cut) was digested with *Dpn*I and *Sal*I endonucleases to remove the input plasmid DNA and to linearize the DNA product while the other aliquot was left at 4 °C. The isolated product was analyzed by electrophoresis on 1% agarose gels in TBE. Details of the procedures are given under Materials and Methods. (A) Gel electrophoretic analysis of the product from the assay of P-4 and S-4 from nontreated (no PEG) NE/S-3. (B) Gel electrophoretic analysis of the product from the assay of P-4 and S-4 from PEG-treated NE/S-3. *Left panel in each figure*: uncut product from incubation with S-4 and P-4 in the presence of T antigen. *Middle panel in each figure*: uncut product from incubation with S-4 and P-4 in the absence of T antigen. *Right panel in each figure*: *Dpn*I and *Sal*I digested (cut) product from incubation with S-4 and P-4 in the presence of T antigen. Arrows indicate form I (closed), form II (nicked, open) monomeric circular, and form III (linear) DNAs.

P-4 is derived (Figure 3A,B, left panel). The products consist of form I and form II DNAs, as well as topological and replicative intermediates. The replication rate with both the NE/S-3 and P-4 fractions is about 25 pmol of dNMP incorporated into *Dpn*I-resistant product h^{-1} (30 ng of DNA)$^{-1}$. This is comparable to replication rates reported by other laboratories for SV40 replication in vitro for crude extracts from HeLa (Li et al., 1985; Wobbe et al., 1985), 293 (Stillman & Gluzman, 1985), and CV-1 (Guo et al., 1989) cells. The synthesis of these products is T-ag dependent (Figure 3A,B, middle panel), and with the P-4 fraction, approximately 85% of the products are *Dpn*I resistant and linearized (form III) following digestion by *Dpn*I/*Sal*I (Figure 3A,B, right panel), suggesting that they result from semiconservative replication (Li & Kelly, 1984; Stillman & Gluzman, 1985). In contrast, when the S-4 fraction, that lacks the sedimentable complex of enzymes for DNA synthesis, from the untreated or PEG-

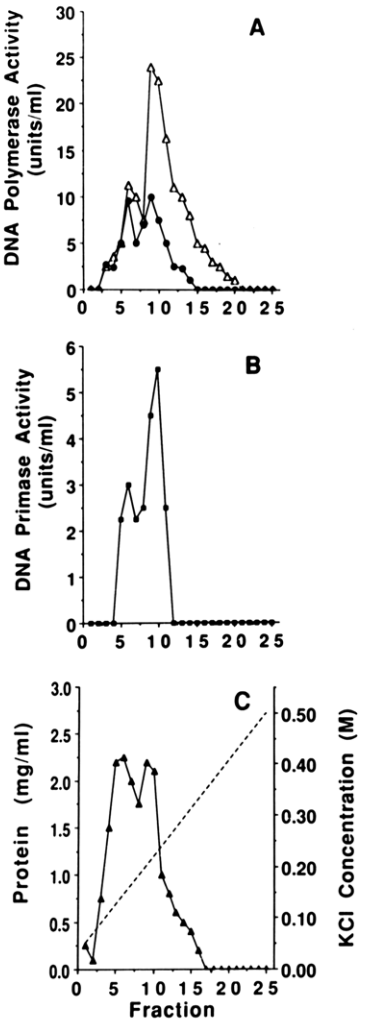

FIGURE 4: Q-Sepharose chromatography of P-4 from the PEG-treated NE/S-3. (A) DNA polymerase activity assayed with (△) activated and (●) primed ssDNA templates. (B) DNA primase activity (■) assayed with poly(dC) template. (C) Protein concentration (milligrams per milliter) (▲) KCl concentration (---) in the continuous gradient from 0.05 to 0.5 M for the elution. Details of the procedures used are described under Materials and Methods.

treated NE/S-3 fraction was used in the incubation in the presence of T-ag, only a small amount of incorporation occurred into form II DNA (Figure 3A,B). This was variable and results from contamination of the nonsedimentable S-4 fraction with some P-4 during the fractionation of the discontinuous gradient.

Q-Sepharose Chromatography. The complex of enzymes that functions in SV40 replication is further purified by chromatography of the P-4 fraction from PEG-treated NE/S-3 on Q-Sepharose at pH 7.5. Approximately 30% of the protein in the P-4 fraction does not bind to the matrix and appears in the column flow-through fraction (data not shown). There is no detectable DNA polymerase, primase, or SV40 in vitro replication activity in the column flow-through fraction, however. Elution of the column with a continuous gradient of increasing KCl concentration from 0.05 to 0.5 M partially resolves two peaks of protein and DNA polymerase α–primase activities that are eluted at KCl concentrations between 0.05 and 0.25 M (Figure 4A–C). The maximum DNA polymerase α activity with activated and primed, ssDNA templates, primase, and 3′,5′ exonuclease activity (Skarnes et al., 1986), resides with the peak that is eluted at a KCl concentration between 0.15 and 0.25 M (Figure 4A,B). DNA-dependent ATPase, DNA ligase, RNase H, and topoisomerase I activities

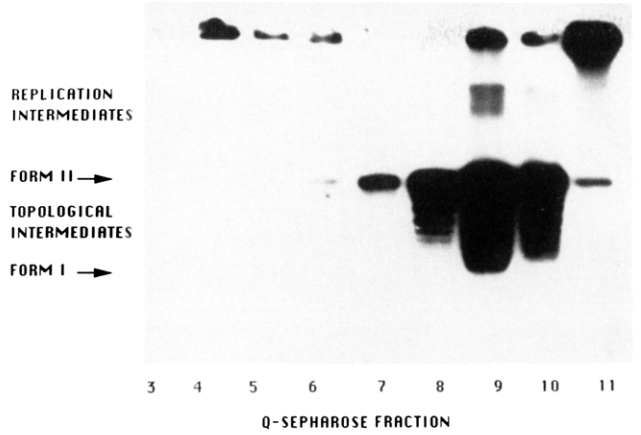

FIGURE 5: Gel electrophoretic analysis of the product from assay of the SV40 in vitro replication activity eluted from Q-Sepharose. Aliquots (16 μg of protein) of the fraction from the eluted peaks of DNA polymerase–primase activities and protein from Q-Sepharose chromatography of P-4 (fractions 3–11 in Figure 4) were incubated for 2 h at 35 °C in the presence of 0.3 μg of T antigen in the assay of SV40 replication activity. Following the incubation, the product was isolated and subjected to gel electrophoretic analysis as described under Materials and Methods.

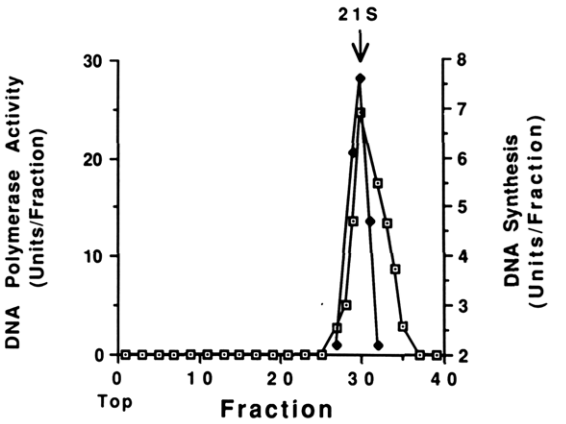

FIGURE 6: Velocity gradient sedimentation analysis of the DNA polymerase activity eluted from Q-Sepharose. 0.1 mL (0.2 mg of protein) of the major eluted peak of DNA polymerase activity (fractions 8–10, Figure 4) from Q-Sepharose chromatography of the P-4 fraction was layered onto a preformed 10–35% glycerol gradient containing 0.5 M KCl prepared in polyallomer tubes for the Beckman SW50.1 rotor. Conditions for centrifugation, fractionation, and assay of DNA polymerase and SV40 replication activity in the gradient were as described under Materials and Methods. (⊡) DNA polymerase activity assayed with activated DNA template and (♦) SV40 in vitro replication activity assayed in the presence of T-ag according to the procedures described under Materials and Methods.

associated with the P-4 fraction also coelute with the protein and DNA polymerase α–primase activities that are eluted by this concentration of KCl (Hickey et al., 1988; data not shown).

The T-ag-dependent SV40 in vitro replication activity in the P-4 fraction also binds to Q-Sepharose and coelutes with the activities of DNA polymerase α–primase, exonuclease, and other enzymes for DNA synthesis that are associated with the enzyme complex. As shown in Figure 5, gel electrophoretic analysis of the product from the assay of the eluted fractions from Q-Sepharose showed that SV40 replication activity (fractions 7–10 in Figure 5) eluted coincidentally with the major peak of DNA polymerase α–primase (fractions 7–12 in Figure 4A,B) and the activities of the other enzymes for DNA synthesis that are eluted at a KCl concentration between 0.15 and 0.25 M from Q-Sepharose. Most of the SV40 replication activity resides in fractions 8–10 (Figure 5). The synthesis is completely dependent on the presence of T-ag in the reaction, and the product consists of monomeric circular form I (closed) and II (nicked) DNAs, as well as topological intermediates and replicative intermediates that migrated more slowly than form II DNA in the gel (fractions 8–10 in Figure 5). Replication intermediates migrating more slowly than form II DNA were much less conspicuous in the gel analysis of the products synthesized by the Q-Sepharose fractions (Figure 5, fractions 8–10) than for the products synthesized by the P-4 fraction (Figure 3A,B, left panel). The products synthesized by fractions eluting on the leading (fraction 7) and trailing (fraction 11) edges of the peak of SV40 replication activity that eluted from Q-Sepharose were form II DNAs and high molecular weight material remaining at the top of the gel (Figure 5, fractions 7 and 11). In the case of fraction 11 at the trailing edge of the eluted peak of SV40 replication activity, the high molecular weight material represented approximately 80% of the *Dpn*I-resistant product synthesized by this fraction. There was no apparent form I DNA, topological or replicative intermediates, synthesized by the eluted fractions 7 and 11 (Figure 5). The products synthesized were *Dpn*I resistant, however, suggesting that they did not arise from repair synthesis. As observed for the synthesis using the cruder P-4 fraction (Figure 3), more than 80% of the products synthesized by the eluted fractions 7–11 from Q-Sepharose chromatography of P-4 (Figure 4) were also *Dpn*I resistant. The replication rate with the peak fractions (8–10) of SV40 replication activity eluted from Q-Sepharose was also similar to that observed for the P-4 fraction, approximately 22 pmol of dNMP incorporated into *Dpn*I-resistant material h^{-1} (30 ng of DNA)$^{-1}$.

Gel electrophoretic analysis of the product from T-ag-dependent synthesis by the fractions within the minor peak of DNA polymerase α–primase activity that is eluted by 0.05–0.13 M KCl during Q-Sepharose chromatography of P-4 (fractions 3–6 in Figure 4A,B) showed mainly synthesis of high molecular weight DNA products (fractions 3–6 in Figure 5). These high molecular weight products, however, were sensitive to *Dpn*I digestion and may result from repair synthesis or intermediates resulting from abortive replication (data not shown).

Through the Q-Sepharose stage of the purification, the DNA polymerase α, primase, and SV40 in vitro replication activities have been purified 100-fold over the activities present in the NE/S-3 fraction and about 400-fold over the activities in the crude cell extract with 70% recovery of the individual activities.

Properties of the 21S Enzyme Complex in SV40 Replication in Vitro

21S Enzyme Complex and SV40 Replication Activity Cofractionate during Q-Sepharose Chromatography. The activities of DNA polymerase α–primase and the other associated enzymes for DNA synthesis and SV40 in vitro replication activity that was eluted within the major peak of these activities during Q-Sepharose chromatography of P-4 (fractions 8–10 in Figures 4A,B and 5) remained associated with 21S enzyme complex during sedimentation analysis on a 10–35% glycerol gradient in the presence of 0.5 M KCl (Figure 6 and data not shown). The replication rate with the 21S enzyme complex after glycerol gradient centrifugation was similar to that observed for the Q-Sepharose fraction, 18 pmol of dNMP incorporated into *Dpn*I-resistant product h^{-1} (30 ng of DNA)$^{-1}$, and about 90% of the products synthesized were converted to form III (linear) DNA following digestion with *Dpn*I/*Sal*I.

The associated activities sediment as a 21S complex during sedimentation analysis in the presence of 0.15 or 0.5 M KCl of the isolated P-4 fraction or the active fractions obtained from Q-Sepharose chromatography of P-4. In contrast, the DNA polymerase activity in the nonsedimentable S-4 fraction, that lacks the 21S enzyme complex and SV40 replication activity, sediments at 7 S under these same conditions (Baril et al., 1988; Hickey et al., 1988). The results from metabolic labeling studies indicate that the 21S enzyme complex obtained from Q-Sepharose chromatography of P-4 is free from detectable nucleic acids.

Proliferating Cell Nuclear Antigen Is Associated with the Enzyme Complex. Proliferating cell nuclear antigen (PCNA) is a 36-kDa, putative cell cycle and growth regulated protein (Celis et al., 1985; Bravo, 1986) that is reported to be an accessory protein of calf thymus DNA polymerase δ (Tan et al., 1986; Prelich et al., 1987a). The results of recent experiments indicate a requirement of PCNA for SV40 replication in vitro (Prelich et al., 1987b; Prelich & Stillman, 1988; Wold et al., 1989; Lee et al., 1989; Weinberg & Kelly, 1989). PCNA was reported to be required for elongation in leading-strand synthesis in SV40 replication in vitro using crude cell extracts (Prelich & Stillman, 1988) and with reconstituted systems (Tsurimoto & Stillman, 1989a; Wold et al., 1989; Lee et al., 1989).

During the course of purification of the 21S complex of enzymes for DNA synthesis, we checked for the presence of PCNA by immunoblot analysis using a monoclonal antibody (19F4) to human PCNA (Ogata et al., 1987). As we had previously observed for DNA ligase, topoisomerase I, and RNase H activities (Hickey et al., 1988), PCNA was detected both with the sedimentable P-4 and with the nonsedimentable S-4 fractions (Figure 7). The P-4-associated PCNA cofractionates with the 21S enzyme complex during each stage of its purification (Figure 7). However, PCNA does not copurify with the 640-kDa, multiprotein DNA polymerase α–primase complex that is resolved from the 21S enzyme complex by sequential steps of chromatography on native and denatured DNA–celluloses and DEAE-Bio-Gel. It is separated from the 640-kDa multiprotein DNA polymerase α–primase complex during chromatography on DEAE-Bio-Gel. The DNA polymerase that is separated from PCNA is not dependent on PCNA for its activity and continues to efficiently replicate primed ssDNA templates including poly(dA)·$(dT)_{10}$ (R. Hickey and E. Baril, unpublished results) as previously reported (Lamothe et al., 1981; Vishwanatha et al., 1986a).

We have not yet shown a requirement for the 21S complex associated PCNA in SV40 replication in vitro. Monoclonal antibody to human PCNA had no effect on the rate of SV40 replication or the product synthesized by the 21S enzyme complex (Li and Baril, unpublished results). More definitive studies such as immunodepletion and reconstitution experiments with purified PCNA and the resolved components of the 21S complex will be required, however, to determine if PCNA functions in SV40 replication by the 21S enzyme complex. These studies will require resolution of the complex into its components and are beyond the scope of this paper.

Multiprotein DNA Polymerase α–Primase Complex Associated with the 21S Enzyme Complex Is Essential for SV40 Replication in Vitro. The results from recent studies have provided indirect evidence that DNA polymerase δ, as well as the DNA polymerase α–primase complex, functions in SV40 replication in vitro [reviewed in Kelly (1988), Kelly et al. (1988), and Stillman (1989)]. Much of the evidence for this comes from reconstitution studies using purified proteins

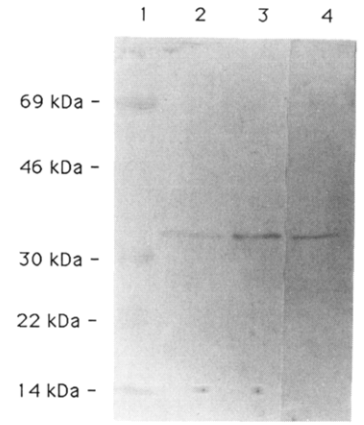

FIGURE 7: Immunoblot analysis for monitoring the presence of PCNA during purification of the 21S enzyme complex. Aliquots of the S-4, P-4, and the eluted fractions from the peak (8–10 in Figure 4) containing DNA polymerase α plus SV40 replication activity from Q-Sepharose chromatography of P-4 were electrophoresed under denaturing conditions on 15% polyacrylamide gels at 30-mA constant current according to a published procedure (Lamothe et al., 1981). Following electrophoresis, the gel was soaked for 1 h in transfer buffer containing 0.01% SDS plus 20% methanol and electrotransferred to a nitocellulose membrane at 20 V for 12 h at 4 °C. After electrotransfer, the membranes were blotted, soaked in blocking buffer for 1 h at room temperature, washed in TBS, and incubated for 3 h with a 1:1000 dilution in TBS of the purified IgG (11.3 mg/mL) fraction from mouse monoclonal antibody to human PCNA (Ogata et al., 1987). The membranes were washed in TBS, incubated for 1 h with a 1:1000 dilution in TBS of alkaline phosphatase conjugated sheep anti-mouse IgG, washed in TBS, and color-developed with 0.005% 5-bromo-4-chloro-3-indolyl phosphate and 0.01% nitroblue tetrazolium. Details of the procedure are given under Materials and Methods. (Lane 1) Protein molecular weight markers ("Rainbow"): BSA (69K), ovalbumin (46K), carbonic anhydrase (30K), trypsin inhibitor (22K), and lysozyme (14K). (Lane 2) S-3 fraction (25 μg of protein). (Lane 3) P-4 fraction (27 μg of protein). (Lane 4) Q-Sepharose of P-4, pooled fractions 8–10 in Figure 4A (20 μg of protein).

and partially characterized fractions of cell proteins (Prelich & Stillman, 1988; Fairman & Stillman, 1988; Lee et al., 1989; Wold et al., 1989; Tsurimota & Stillman, 1989a; Weinberg & Kelly, 1989). DNA polymerase δ was originally distinguished from other cellular DNA polymerases on the basis of its tightly associated 3′,5′ exonuclease activity and its PCNA requirement for synthesis with primed, ssDNA templates such as poly(dA)·oligo(dT) (So & Downey, 1988). DNA polymerase δ is also reported to be immunologically (So & Downey, 1988; Wong et al., 1989) and structurally (Wong et al., 1989) distinct from DNA polymerase α and much less sensitive to the DNA polymerase α inhibitor BuPdGTP (Byrnes, 1985; Lee et al., 1985).

In earlier studies, we showed the presence of a 640-kDa protein band in immunoblot analysis of P-4 using monoclonal antibody SJK 132-20 (Tanaka et al., 1984) to proteolytic fragments of the human 180-kDa DNA polymerase α catalytic subunit (Hickey et al., 1988). A 640-kDa band was not observed, however, in immunoblots of S-4 performed under the same conditions. In those experiments, the P-4 and S-4 fractions were electrophoresed under nondenaturing conditions that are known to preserve the native structure of the 640-kDa multiprotein DNA polymerase α–primase complex (Lamothe et al., 1981; Vishwanatha et al., 1986a). After electrotransfer of the protein to nitocellulose, immunoblot analysis was performed with the antibody to human polymerase α (Hickey et al., 1988). The existence of the 640-kDa multiprotein DNA polymerase α–primase complex with the P-4 but not the S-4 fraction was corroborated by fractionating the P-4 and S-4 fractions through succesive steps of chromatography on

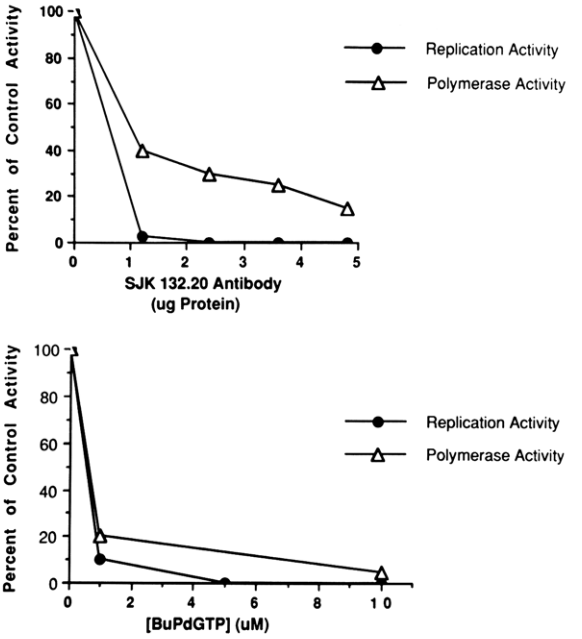

FIGURE 8: Inhibition of SV40 in vitro replication and DNA polymerase α activities associated with the enzyme complex by monoclonal antibody (SJK 132-20) to human polymerase α and BuPdGTP. (Upper panel) Aliquots of the isolated P-4 fraction (32 μg of protein) were preincubated at 4 °C for 1 h in the presence of the designated amounts of SJK 132-20 purified IgG. Following the preincubation, the remaining components for the SV40 replication assay were added to some of the preincubated tubes, and activated DNA plus the other components for the polymerase α assay was added to the remaining tubes. Incubation was continued at 35 °C according to the procedure described under Materials and Methods. (●) SV40 replication activity; (△) DNA polymerase α activity. Control activities for SV40 replication and DNA polymerase α equal 0.10 and 0.51 units, respectively. (Lower panel) The P-4 fraction was incubated with activated DNA template under the conditions for DNA polymerase α assay or with components for the SV40 in vitro replication assay and with the designated amounts of BuPdGTP according to the procedure described under Materials and Methods. (●) SV40 replication activity; (△) DNA polymerase α activity. Control activities for SV40 replication and DNA polymerase α activities equal 0.10 and 0.50 units, respectively.

DEAE-cellulose, native and denatured DNA–cellulose, and DEAE-Bio-Gel using a procedure developed for the isolation of the 640-kDa multiprotein polymerase α–primase complex (Vishwanatha et al., 1986a). The DNA polymerase α complex isolated from P-4 is 10S and has the physical and enzymatic properties of the previously described 640-kDa multiprotein polymerase–primase complex (Vishwanatha et al., 1986a) while the DNA polymerase isolated from S-4 is 7S and lacks properties of the 640-kDa multiprotein polymerase α–primase complex (Baril et al., 1988).

As shown in Figure 8, the DNA polymerase activity associated with the P-4 fraction is completely inhibited by monoclonal antibody SJK 132-20 to human polymerase α (Tanaka et al., 1982). The antibody completely inhibits the DNA polymerase activity assayed with primed ssDNA, as well as with activated DNA as template (Malkas, Li, Hickey, and Baril unpublished results). This antibody also completely inhibits the T-ag-dependent SV40 in vitro replication activity that is exclusively associated with the P-4 fraction containing the 21S enzyme complex. Complete inhibition of the SV40 replication activity, however, occurs at a lower IgG concentration than is required for complete inhibition of the polymerase activity (Figure 8).

BuPdGTP inhibits both DNA polymerase α and DNA polymerase δ activity although the activity of the latter enzyme is much less sensitive to the nucleotide analogue (Byrnes, 1985;

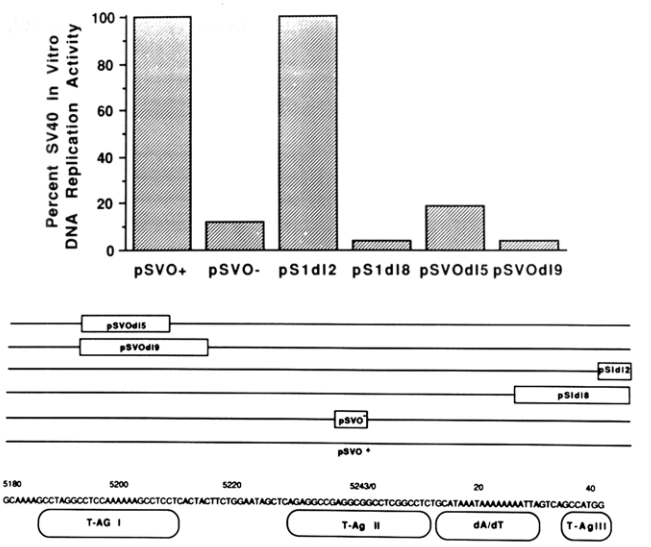

FIGURE 9: Comparison of the in vitro replication activity of the enzyme complex with the plasmid containing SV40 ori (PSVO$^+$) to plasmids with deletion-containing SV40 ori DNA inserts. The assay of in vitro replication activity was performed with the P-4 fraction (35 μg of protein) in the presence of 0.3 μg of T-ag and the reaction terminated by precipitation with 10% TCA and filtration on GF/C glass fiber filters as described under Materials and Methods. Hatched bars represent percent in vitro replication activity relative to the activity with SV40 ori containing plasmid pSVO$^+$ (100%). The 100% value equals 0.16 unit. In the scheme of the minimal core SV40 ori (lower part of figure), the T-ag blocks designate sequences for T antigen binding sites I, II, and III while the dA/dT block designates sequences of the 17 bp A/T-rich tract in the initiation zone of SV40 core ori. The horizontal lines represent the SV40 ori DNAs used in the assays, and pSVO$^+$ designates the complete SV40 ori DNA insert. The rectangular boxes represent the deleted sequences, and the designation of the deletion DNA is given within the box. The nucleotide sequences (inclusive) deleted in each of the DNAs is as follows: pSVO$^-$ (5239–5242), pSVOd15 (5192–5208), pSVOd19 (5192–5216), pSId18 (23–56), and pS1d12 (40–56).

Lee et al., 1985). DNA polymerase α activity is completely inhibited at BuPdGTP concentrations of 0.1–10 μM while complete inhibition of DNA polymerase δ activity requires about 100-fold higher concentrations. As shown in Figure 8, DNA polymerase α and the in vitro SV40 replication activity associated with the P-4 fraction are both completely inhibited by BuPdGTP at concentrations of 10 and 5 μM, respectively (Figure 8). These concentrations of BuPdGTP are 50–100-fold lower than that reported to be required for complete inhibition of DNA polymerase δ activity (Byrnes, 1985).

Taken together, these results suggest that the predominant DNA polymerase activity associated with the 21S enzyme complex belongs to the α class of DNA polymerases and that its activity is essential for SV40 replication in vitro.

SV40 Replication by the 21S Enzyme Complex Requires Functional Origin Sequences. A functional origin (ori) sequence is required for effective replication of SV40 DNA in vitro in cell-free extracts, as well as in vivo (Stillman et al., 1985; Li et al., 1986). The boundaries of the minimal core origin of replication, as defined from in vivo [reviewed in DePamphilis and Wassarman (1980)] and in vitro (Li et al., 1986) replication analysis with deletion mutants, exist in a 65 bp segment (nucleotides 5208–5230) of the SV40 genome. As diagrammed in Figure 9, the minimal core ori includes T antigen binding site II (T-Ag II), a section of T antigen binding site I (T-Ag I), and a 17 bp A/T-rich tract (nucleotides 5215–5231 in Figure 9) that lies in the initiation zone of SV40 ori (Yamaguchi & DePamphilis, 1986) and gives rise to a bent DNA region that is essential for the initiation of SV40 replication in vivo and in vitro (Deb et al., 1986; Hertz et al.,

1987). The minimal core origin is approximately centered on a 27 bp palindrome that includes the T-Ag II binding site (Figure 9, nucleotides 5230–13) and a 15 bp imperfect inverted repeat (nucleotides 5193–5207) within the T-Ag I binding site (Li et al., 1986).

Deletion mutant DNAs (Stillman et al., 1985) having sequences deleted from the late (pS1 series) or early (pSVO series) mRNA side of the T antigen II binding site in the SV40 ori were used as templates for the analysis of the sequence requirements for initiation of SV40 replication in vitro by the 21S enzyme complex (Figure 9). Deletions in ori on the early mRNA side of the T-Ag II binding site that included removal of the inverted repeat sequence within the T-Ag I binding site (pSVOd15 and pSVOd19 DNAs) reduced the initiation of DNA synthesis in vitro by 80–95% (Figure 9). A 4 bp deletion within the palindrome at the T-Ag II binding site ($pSVO^-$ DNA) reduced the efficiency of initiation of replication by the enzyme complex approximately 90%. Deletions on the late mRNA side of the T-Ag II binding that resulted in the removal of sequences within the A/T-rich tract (pS1d18) also reduced the effectiveness of the template for in vitro initiation by the enzyme complex by 95% or more (Figure 9). In contrast, the efficiency of initiation of the mutant DNA containing a deletion of sequences to the late side of T-Ag II binding site, but outside of the boundary of the minimal core ori (pS1d12 DNA), was the same as for the wild-type ($pSVO^+$) DNA (Figure 9).

These results with the isolated 21S enzyme complex are comparable to those reported for the replication in vivo and in vitro of an extensive list of deletion mutant SV40 DNAs that included the deletion DNAs used in this study (Stillman et al., 1985; Li et al., 1986, Guo et al., 1989). In the earlier studies, it was also found that the sequences that constitute the minimal core ori are necessary for the efficient initiation of SV40 replication in vivo and in vitro (Stillman et al., 1985; Li et al., 1986).

Auxiliary sequences that flank the minimal core ori are known to facilitate the initiation of SV40 replication in vivo (DeLucia et al., 1986; Hertz & Mertz, 1986; Li et al., 1986; Guo et al., 1989). By optimizing the conditions of the in vitro SV40 replication assay for sensitivity to auxiliary sequences, Guo and co-workers (Guo et al., 1989) recently demonstrated that origin auxiliary sequences facilitate the initiation of SV40 replication in vitro, as well as in vivo. The assay conditions used in our study were not optimized for sensitivity to auxiliary sequences, and the plasmid DNAs employed lacked the complete auxiliary 2 sequences, nucleotides 30–72 (DeLucia et al., 1986; Guo et al., 1989), to the late mRNA side of T-Ag II (Figure 9). Thus, an answer to the important question of the influence of auxiliary sequences on the initiation of SV40 replication by the 21S enzyme complex must await further investigation.

Discussion

The major conclusion from this study is that the SV40 in vitro replication activity in HeLa cell extracts resides with a sedimentable 21S complex that contains requisite enzymes and nonenzymic proteins for DNA synthesis. The enzyme complex that is recovered with the sedimentable P-4 subfraction of the NE/S-3 fraction contains most of the DNA polymerase α–primase complex, DNA-dependent ATPase, and RNase H and essentially all of the T-ag and ori-dependent SV40 in vitro replication activity present in the HeLa cell homogenate (Baril et al., 1988; Hickey et al., 1988). The individual enzymes and SV40 replication activity remain associated with the 21S complex during the course of its purification.

There are a number of lines of evidence that indicate that the 21S enzyme complex does not arise from nonspecific aggregation or adsorption. First, the enzyme complex and also the SV40 replication activity exist as a sedimentable 21S complex at all stages of the purification including anion-exchange chromatography using conventional chromatographic or FPLC procedures. The associated enzymes and SV40 replication activity remain associated during PEG precipitation in the presence of 2 M KCl and cosediment as a 21S complex during velocity gradient sedimentation under conditions of moderate (0.15 M KCl) or high (0.5 M KCl) ionic strength. The results of studies to be published elsewhere (Li and Baril, unpublished results) demonstrate that the 21S enzyme complex can be isolated directly from the combined NE/S-3 or S-3 fractions without the PEG precipitation or discontinuous sucrose gradient centrifugation steps to isolate P-4. The enzyme complex isolated by this procedure also sediments at 21 S at all stages of its purification including FPLC and retains the same complement of enzymes for DNA synthesis and essentially all of the SV40 replication activity present in the homogenate of HeLa cells. Finally, some of the activities of enzymes for DNA synthesis (e.g., the 640-kDa multiprotein DNA polymerase α–primase complex, DNA-dependent ATPase, SV40 ori-specific dA/dT binding protein, and ori-specific, T-ag-dependent SV40 replication) are associated almost exclusively with the 21S complex while others (e.g., topoisomerase I, DNA ligase, PCNA, RNase H, and DNA polymerase α) are present both with the 21S enzyme complex and with the soluble S-4 fraction. SV40 replication activity, however, resides only with the sedimentable 21S complex of enzymes for DNA synthesis and not with the S-4 fraction containing soluble enzymes. It seems unlikely that a complex of enzymes that survives several purification steps while retaining its physical size of 21S and the enzymatic machinery required for SV40 replication results from nonspecific aggregation or adsorption of soluble enzymes and nonenzymic proteins in the cell extract.

The intracellular origin of the 21S complex of enzymes for DNA synthesis reported here is unknown at this time. The enzyme complex is extracted from HeLa cells under the usual low ionic strength conditions that are employed for the extraction of SV40 in vitro replication activity (Li et al., 1984; Stillman & Gluzman, 1985; Wobbe et al., 1985; Yamaguchi & DePamphilis, 1986). These conditions are milder than the conditions required for the extraction of chromatin or nuclear matrix associated proteins. Also, the results of metabolic labeling experiments indicate that the enzyme complex isolated through the Q-Sepharose chromatography stage of its purification has no detectable DNA or RNA associated with it. Although it appears unlikely that the isolated 21S enzyme complex is associated with chromatin or nuclear matrix, we cannot rule out the possibility that the complex may be associated with such structures in the cell. The inclusion of PEG in the incubation system was reported to enhance the activity of CV-I cell extracts for replication of SV40 minichromosomes in vitro (Decker et al., 1987). The PEG precipitation step employed in our purification, however, does not appear to contribute to the formation of the 21S enzyme complex since the complex can be isolated directly from the combined NE/S-3 without PEG precipitation (Li and Baril, unpublished results).

There have been several reports over the past decade of the isolation of enzyme complexes for DNA synthesis from eukaryotic cells [reviewed in Fry and Loeb (1986) and Mathews and Slabaugh (1986)]. Reddy and Pardee (1980) isolated a

DNA-associated complex of enzymes for DNA synthesis from hamster fibroblast (CHEF) cells that they designated replitase. Tubo and Berezney (1987) reported the isolation of 100–150S megacomplexes containing DNA polymerase α–primase and other enzymes for DNA synthesis associated with the nuclear matrix of regenerating rat liver. No direct involvement of the replitase or the 100–150S megacomplexes in DNA replication was demonstrated, however, probably due to the lack of functional assays. Jackson and Cook (1986) did observe the retention of megacomplexes containing DNA polymerase α and other enzymes for DNA synthesis in agarose-entrapped nuclei from HeLa cells. The enzyme megacomplexes, but not their dissociated counterparts, efficiently replicated endogenous chromosomal DNA in nuclei. Jazwinski and Edelman (1984) also reported the isolation of a 2 million dalton enzyme complex from yeast cells that had associated DNA polymerase I (the yeast counterpart of polymerase α), primase, DNA ligase, and topoisomerase II activities. The 2 million dalton complex efficiently replicated the yeast 2-μm plasmid in vitro. Thus, there is evidence for an interaction of enzymes for DNA synthesis in eukaryotes to form functional DNA synthesizing complexes. The advent of an in vitro system for SV40 replication (Li & Kelly, 1984) has provided a functional assay for the identification and analysis of such complexes isolated from permissive mammalian cells.

The requirements for SV40 replication in vitro by the isolated 21S enzyme complex reported here are comparable to the requirements that have been observed with crude extracts from permissive cells. Namely, the initiation of SV40 replication is dependent on the presence of both T-ag (Li & Kelly, 1984; Stillman & Gluzman, 1985; Wobbe et al., 1985; Yamaguchi & DePamphilis, 1986; Figure 3 of this paper) and functional SV40 core ori sequences (Stillman et al., 1985; Li et al., 1986; Figure 9 of this paper) in the reaction. The replication rate with the isolated enzyme complex, approximately 18–25 pmol of dNMP incorporated into *Dpn*I-resistant product h^{-1} (30 ng of DNA)$^{-1}$, remains about the same throughout its isolation and is comparable to the rate observed by others for SV40 replication with crude cell extracts (Li et al., 1985; Gluzman & Stillman, 1985; Wobbe et al., 1985; Guo et al., 1989). Among the products synthesized by the enzyme complex are form I and form II DNAs, as well as topological and replicative intermediates. These products are synthesized throughout the course of purification of the 21S complex, and the majority of the synthesized products (i.e., 80–90% or more) are resistant to *Dpn*I, that digests the methylated plasmid DNA, and are linearized by *Sal*I, that acts at a single cleavage site in the plasmid DNA (Li & Kelly, 1984). These results are consistent with semiconservative replication of full-length DNA as has been observed for T-ag-dependent SV40 replication in cell-free extracts (Li & Kelly, 1984; Gluzman & Stillman, 1985; Wobbe et al., 1985; Yamaguchi & DePamphilis, 1985). The products synthesized by the 21S enzyme are similar to the products synthesized by the starting crude NE/S-3 fraction from the HeLa cell homogenate (Figures 3 and 5; Baril et al., 1988). These products are also similar to the products that were found to be synthesized in SV40 replication with crude cell extracts (Li & Kelly, 1984, 1985; Stillman & Gluzman, 1985; Wobbe et al., 1985; Yamaguchi & DePamphilis, 1986). Finally, some of the enzymes and nonenzymic proteins associated with the 21S complex (i.e., DNA polymerase α–primase complex, DNA ligase, RNase H, topoisomerase I, and PCNA) have also been reported to participate in SV40 replication in vitro (Murakami et al., 1986; Ishimi et al., 1988; Wold et al., 1989; Tsurimoto & Stillman, 1989a,b). Taken together, these results indicate that the existence of the 21S enzyme complex reported here is not fortuitous and that it probably has a role in DNA replication.

The 21S enzyme complex has been shown (Baril et al., 1988; Hickey et al., 1988) to contain the activities of some enzymes (e.g., DNA polymerase α–primase complex, DNA ligase, RNase H, and topoisomerase I) and other proteins, e.g., the ori-specific dA/dT sequence binding protein (Malkas & Baril, 1989) and PCNA, that probably function in DNA replication. However, additional proteins are present with the complex whose function, if any, in DNA replication remains to be defined. For example, the results from in vitro reconstitution experiments indicate that a host cell single-stranded DNA binding protein (Wobbe et al., 1987; Fairman & Stillman, 1988; Wold & Kelly, 1988) and also PCNA and DNA polymerase δ (Fairman & Stillman, 1988; Tsurimoto & Stillman, 1989a,b; Wold et al., 1989; Lee et al., 1989; Weinberg & Kelly, 1989) are required for the elongation stage in SV40 replication in vitro. Also to be defined is the basis for the formation of form I DNA among the products synthesized by the 21S enzyme complex. The synthesis of form I DNA has not generally been observed in reconstitution experiments for SV40 replication in vitro with purified proteins and partially purified cell fractions (Wobbe et al., 1985, 1987; Ishimi et al., 1988) but was observed among the products synthesized by crude cell extracts (Li & Kelly, 1984, 1985; Wobbe et al., 1985). The addition of small amounts of crude cell extract to the reconstituted system was required in order to generate form I DNA (Ishimi et al., 1988). Stillman (1986) showed that the synthesis of form I DNA by the cytosol fraction from 293 cell extracts was dependent on the addition of a low-salt extract of sonicated cell chromatin to the cytosol fraction. The low-salt chromatin extract promoted chromatin assembly during SV40 replication in vitro by the cytosol fraction that resulted in the formation of the negatively supercoiled form I DNA. We observe the synthesis of form I DNA among the products synthesized by the low-salt nuclear extract (that is not a chromatin extract), the cytosol fraction, and P-4 (Baril et al., 1988) as well as with the purified 21S enzyme complex (Figure 5). The form I DNA is converted to a relaxed form by treatment with purified HeLa cell topoisomerase I, suggesting that it does not exist in a nucleoprotein structure. We have no evidence that histones are present with the purified 21S enzyme complex. However, further investigation is required to define the basis for the formation of form I DNA among the products synthesized by the enzyme complex. Also calling for further investigation is the nature of the factors involved in the processing of the high molecular products synthesized by the 21S enzyme complex into monomeric, circular-replicated molecules. Topoisomerase II is required for segregation of the replicated daughter molecules in SV40 replication in vivo [reviewed in DePamphilis and Wasserman (1980), Kelly et al. (1988), and Stillman (1989)] and for decatenating the newly synthesized daughter molecules in SV40 replication in vitro (Yang et al., 1987). We observe very low (barely detectable) levels of topoisomerase II activity in the NE/S-3 and P-4 fractions from HeLa cell extracts using the sensitive unknotting assay (Miller et al., 1981) with *Crithidia* kinetoplast DNA as substrate (Baril et al., 1988; Hickey et al., 1988). There is a significant amount of *Dpn*I-resistant monomeric circular form I and form II DNAs among the products synthesized by these fractions, however (Figure 3; Baril et al., 1988). Although there is an accumulation of *Dpn*I-resistant high molecular product synthesized by the purified enzyme complex (Figure 5), a significant

amount of monomeric circular DNA product is synthesized, suggesting the presence of topoisomerase II with the complex. We cannot explain at this time the low topoisomerase II activity that is detected with these fractions. Further studies are required to determine if this is due to the presence of inhibitors, modification of the topoisomerase with these fractions that alters its detection, or other reasons that need to be unraveled.

Antibody to human polymerase α (Tanaka et al., 1982) and BuPdGTP, at concentrations that selectively inhibit DNA polymerase α activity, completely inhibit both the polymerase α and SV40 replication activity associated with the 21S enzyme complex (Figure 8). These results corroborate earlier evidence obtained through immunodepletion (Murakami et al., 1986) and in vitro reconstitution experiments (Ishimi et al., 1988; Wold et al., 1989) that show that the DNA polymerase α–primase complex is essential for the initiation of SV40 replication in vitro. The results of biochemical and immunological analysis (Baril et al., 1988; Hickey et al., 1988) indicate that the DNA polymerase α–primase complex associated with the 21S enzyme complex resides with the previously described 640-kDa multiprotein DNA polymerase α–primase complex (Lamothe et al., 1981; Vishwanatha et al., 1986a). The association of DNA polymerase α–primase in the 640-kDa multiprotein complex was previously shown to profoundly affect the selection of template initiation sites and the length of primers synthesized by the polymerase α–primase complex on ss SV40 DNA inserts containing ori sequences (Vishwanatha et al., 1986b). The 640-kDa polymerase α–primase is probably a key component of the 21S enzyme complex since biochemical and immunological analysis indicates that virtually all (more than 95%) of the 640-kDa polymerase α–primase complex in HeLa cell homogenates resides with the sedimentable 21S enzyme complex (Baril et al., 1988; Hickey et al., 1988). The 640-kDa polymerase α–primase complex is larger than the four-polypeptide DNA polymerase α–primase complex that was originally purified from *Drosophila melanogaster* embryos [reviewed in Kaguni and Lehman (1988) and Lehman and Kaguni (1989)] and more recently from mouse cells (Goulian & Heard, 1989). It is, however, similar in size and polypeptide structure to a multiprotein DNA polymerase α–primase complex that was purified from calf thymus by Ottiger and co-workers (Ottiger et al., 1987). In addition to the DNA polymerase α catalytic subunit and primase, the 640-kDa polymerase α–primase from HeLa cells (Vishwanatha et al., 1986a) has an associated 69-kDa 3′,5′ exonuclease with low but detectable 5′,3′ exonuclease activity (Skarnes et al., 1986) and the C_1C_2 primer recognition proteins (Lamothe et al., 1981; Pritchard & DePamphilis, 1983; Pritchard et al., 1983; Vishwanatha et al., 1986a). The latter proteins permit the DNA polymerase α to function efficiently with ssDNA templates of low primer to template ratios (Lamothe et al., 1981; Pritchard et al., 1983; Vishwanatha et al., 1986a). A multiprotein factor (RF-C) that is essential for elongation in a reconstituted system for SV40 replication in vitro was isolated from human 293 cells (Tsurimoto & Stillman, 1989a). RF-C was shown to affect the processivity of DNA synthesis and the frequency of primer recognition by DNA polymerases α and δ on poly(dA)·oligo(dT) (Tsurimoto & Stillman, 1989b). In the presence of PCNA and a multiprotein DNA binding factor (RF-A), RF-C stimulated DNA polymerase α activity 4–6-fold and polymerase δ 25–30-fold on ss M13 DNA template primed at a single site. The relationship, if any, of the C_1C_2 primer recognition proteins associated with the 640-kDa polymerase α–primase complex from HeLa cells to the multiprotein RF-C of 293 cells (Tsurimoto & Stillman, 1989a,b) remains to be defined.

DNA polymerase δ has also recently been implicated in SV40 replication in vitro (Prelich et al., 1987a,b; Fairman & Stillman, 1988; Lee et al., 1989; Weinberg & Kelly, 1989), and PCNA is reported to be an accessory protein for DNA polymerase δ (Tan et al., 1986). Much of the evidence for the participation of DNA polymerases α and δ in SV40 replication has come from in vitro reconstitution experiments using purified proteins, as well as partially characterized cell protein fractions (Fairman & Stillman, 1988; Tsurimoto & Stillman, 1989a,b; Lee et al., 1989; Weinberg & Kelly, 1989). Although direct evidence for the involvement of DNA polymerase δ is still lacking, models for the participation of DNA polymerases α and δ in SV40 replication have been proposed (Fairman & Stillman, 1988; So & Downey, 1988; Stillman, 1989; Lee et al., 1989). In general, these models depict DNA polymerase α–primase functioning in the initiation of leading- and lagging-strand synthesis at the replication fork while DNA polymerase δ functions in the elongation step of SV40 replication. PCNA-dependent (Lee et al., 1989) and PCNA-independent (Syvaoja & Linn, 1989) forms of polymerase δ have been reported to exist in HeLa cells. Our repeated efforts, however, to define the presence of DNA polymerase δ, as well as DNA polymerase α, with the NE/S-3 and P-4 fractions or the purified 21S enzyme complex from HeLa cells that efficiently replicate SV40 DNA in vitro have failed. In the results of studies to be published elsewhere (Li, Malkas, Hickey, and Baril, unpublished results), we observed that during immunoaffinity chromatography of the NE/S-3, P-4, or the isolated 21S complex from HeLa cells, more than 95% of the DNA polymerase activity assayed under optimal conditions for DNA polymerase α or δ remained bound to the agarose-linked IgG for monoclonal antibodies SJK 237 or SJK 132-20 to human polymerase α (Tanaka et al., 1982). The small amount of DNA polymerase activity (5% or less) appearing in the column flow-through fraction was completely inhibited by the neutralizing antibody SJK 132-20 to human polymerase α (Li, Malkas, Hickey, and Baril, unpublished results). This is similar to what we had observed for the polymerase activity associated with P-4 and the isolated 21S complex (Figure 8; Baril et al., 1988; Hickey et al., 1988). As discussed earlier, the DNA polymerase activity that is isolated from the 21S enzyme complex by the conventional chromatographic procedure previously described (Vishwanatha et al., 1986a) is also completely inhibited by the neutralizing antibody SJK 132-20 to human polymerase α when assayed under optimal conditions for DNA polymerase α and δ activities. Immunoblot analysis with monoclonal antibody (19F4) to human PCNA (Ogata et al., 1987) indicated that the DNA polymerase activity that is resolved from the 21S does not contain associated PCNA and does not require PCNA to function efficiently with primed ssDNA templates such as poly(dA)·oligo(dT) (Hickey and Baril, unpublished results). We have no explanation at this time for the apparent discrepancy of our results for the absence of detectable DNA polymerase δ activity associated with the isolated 21S enzyme complex that efficiently replicates SV40 DNA in vitro and the reported participation of DNA polymerase δ in SV40 replication in vitro in reconstitution experiments with proteins and protein-containing fractions from HeLa cells (Lee et al., 1989; Weinberg & Kelly, 1989). Further studies that include direct reconstitution experiments with the components of the 21S enzyme complex are required in order to resolve these differences. These studies are being undertaken in our laboratory.

The 21S complex of enzymes for DNA synthesis from HeLa cells, in results that will be published elsewhere, has recently been extensively purified with full retention of its ability to efficiently replicate SV40 DNA in vitro (Li and Baril, unpublished results). The purified complex is composed of approximately 25 polypeptides. Further characterization of this complex in regard to its organization and the functional interaction of its components in SV40 replication should provide insights into the organization of the DNA synthesizing apparatus for chromosomal DNA replication in mammalian cells.

ACKNOWLEDGMENTS

The excellent assistance of Joan Golrick in the tissue culture experiments and Sandra Johnson in the preparation of the manuscript is gratefully acknowledged. We are also appreciative to Drs. Y. Gluzman and B. Stillman for plasmids containing deletions in the SV40 origin, Drs. D. Weinberg and T. Kelly for the recombinant virus AdSVR284, Dr. G. Wright for providing the BuPdGTP used in this study, and Dr. David Kupfer for advice on the poly(ethylene glycol) precipitation procedure.

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A Bispecific Antibody Enhances the Fibrinolytic Potency of Single-Chain Urokinase†

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Received August 30, 1989; Revised Manuscript Received January 24, 1990

ABSTRACT: A monoclonal antibody specific for an epitope at the amino terminus of the β chain of fibrin and a monoclonal antibody that binds both one- and two-chain high molecular weight urokinase were chemically cross-linked [using *N*-succinimidyl 3-(2-pyridyldithio)propionate and 2-iminothiolane]. The chemically modified material was heterogeneous, ranging in molecular size from tetramers to monomers and containing the two antibodies in various ratios. Nevertheless, fractions of a molecular size larger than a monomer were capable of binding fibrin and urokinase simultaneously in a radioimmunoassay. These fractions also enhanced fibrinolysis by high molecular weight single-chain urokinase (scuPA) by 50-fold and plasma clot lysis by 5-fold. Whereas scuPA significantly decreased the concentration of fibrinogen in plasma clot assay supernatants, scuPA in association with the bispecific antibody did not.

Tissue plasminogen activator (tPA)[1] and single-chain urokinase plasminogen activator (scuPA) interact with plasminogen to convert it to the active enzyme plasmin. Plasmin then lyses thrombi by degrading fibrin. Bleeding complications arise because at the doses required for the prompt lysis of thrombi the generation of excess plasmin may result in the degradation of fibrinogen, α_2-antiplasmin, factors V and VIII, and the platelet GPIIb/IIIa receptor complex that binds fibrinogen (Bennett et al., 1982; Verstraete & Collen, 1986). The degradation of these proteins (and others important in hemostasis) can induce a systemic lytic state. Increasing the fibrin specificity of plasminogen activators has been proposed as a mechanism by which plasmin generation at the site of a thrombus can be enhanced and systemic activation of the fibrinolytic system can be avoided (Collen et al., 1989; Dewerchin et al., 1989; Haber et al., 1989; Maksimenko & Torchilin, 1985; Nakayama et al., 1986; Robbins & Boreisha, 1987). The high affinity and specificity of antifibrin monoclonal antibodies makes them suitable agents for further increasing the fibrin specificity of plasminogen activators such as tPA and scuPA.

In this paper, we describe the preparation and biochemical characterization of a bispecific (antifibrin–antiurokinase) antibody. A high-affinity antifibrin monoclonal antibody, 59D8 (Hui et al., 1983), was chemically coupled to an antiurokinase monoclonal antibody, PEG2, that had been produced

† Supported in part by National Institutes of Health Grants HL-19259 and HL-28015.
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[1] Abbreviations: tPA, tissue plasminogen activator; scuPA, high molecular weight single-chain urokinase; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate.

0006-2960/90/0429-6374$02.50/0