DNA Replication Machinery: Functional Characterization of a Complex Containing DNA Polymerase α, DNA Polymerase δ, and Replication Factor C Suggests an Asymmetric DNA Polymerase Dimer†

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ABSTRACT: By using a complementation assay for a replication factor C dependent DNA polymerase activity on a singly-primed M13 DNA template, we have isolated from calf thymus a multiprotein complex active in DNA replication. For this, the inclusion of ATP during the entire isolation procedure was essential, since the complex decayed after omission of ATP. This complex contains at least DNA polymerase α/primase, DNA polymerase δ, and replication factor C as shown by gel-filtration and coimmunoprecipitation experiments. It is functionally active in replication of primed and unprimed single-stranded M13 DNA templates. Furthermore, in the presence of proliferating cell nuclear antigen and ATP, it forms an isolatable holoenzyme/template-primer complex. Replication factor C apparently mediates the interaction of DNA polymerase δ in the complex with proliferating cell nuclear antigen, through an ATP-dependent mechanism. This interaction appears to stabilize the binding of the complex to a template-primer and to coordinate the activity of DNA polymerase α/primase and DNA polymerase δ during replication of a single-stranded DNA template. Our data suggest the existence of an asymmetric DNA polymerase complex in mammalian cells.

DNA replication requires the coordinated action of several proteins and enzymes, acting either separately or in a complex. The factors required for DNA replication include DNA polymerases (pols)[1] and their accessory proteins, DNA primase, DNA topoisomerases, DNA helicases, DNA binding proteins, RNase, DNA ligases, and other factors conferring specificity at the origins of DNA replication [reviewed in Hübscher and Spadari (1994) and Stillman (1994)]. The functions of these proteins have been established by reconstitution experiments *in vitro* starting from purified components either in prokaryotic or in eukaryotic systems. By using ss plasmid DNA of φX174, M13, or G4 bacteriophages as model replicons, the requirement for a SSB and a DNA synthesis complex was first identified in prokaryotes. This complex in *Escherichia coli* is called pol III holoenzyme and consists of the pol III core, the homodimeric β subunit, and the heteropentameric γ complex (Maki et al., 1988). Similarly, in bacteriophage T4, the product of gene 45 and the complex of the products of genes 44/62 perform functions analogous to the β subunit and the γ complex, respectively [reviewed in von Hippel et al. (1992)]. The function of such a replication-competent (RC) multiprotein complex is to produce an accurate copy of the genome to be replicated. In order to achieve this goal, replication-specific pols must execute several functions other than that of polymerization of dNTPs. These include cooperation in unwinding of the parental DNA strands, coordination with primase-dependent synthesis of RNA primers, continuous elongation of the leading strand and discontinuous synthesis on the lagging strand, processive translocation along the template, and production of a faithful complementary copy of the parental template. This implies that a replication-specific pol must be able to cooperate with the other enzymes and proteins involved in DNA replication.

DNA replication in eukaryotic cells is thought to be carried out by three distinct pols, namely, pol α, pol δ, and pol ε [reviewed in Hübscher and Spadari (1994) and Stillman (1994)]. In addition, at least three pol accessory proteins, PCNA, RF-C, and RP-A, appear to be part of the DNA replication machinery. They are the eukaryotic counterparts of the *E. coli* pol III β subunit (PCNA), γ complex (RF-C), and SSB (RP-A), respectively [reviewed in Hübscher et al. (1996)]. Their roles have been elucidated by *in vitro* reconstitution of SV40 DNA replication with purified components (Waga et al., 1994; Waga & Stillman, 1994). First, unwinding of the origin of replication occurs by the combined action of TAg and RP-A; then pol α/primase initiates synthesis at the origin (Wold et al., 1987; Lee et al., 1989; Tsurimoto et al., 1989; Borowiec et al., 1990). Being associated with DNA primase activity, pol α is involved in the primer synthesis and in the partial elongation of the newly formed RNA primers (Boroweic et al., 1990; Tsurimoto et al., 1990). After pol α/primase synthesizes short stretches of DNA, PCNA and RF-C form a tight complex in the presence of ATP at the 3′-OH end of the

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[1] Abbreviations: pol, DNA polymerase; SV40, simian virus 40; TAg, SV40 large T-antigen; PCNA, proliferating cell nuclear antigen; RF-C, replication factor C; RP-A, replication protein A; SSB, *E. coli* single-stranded DNA binding protein; KF, Klenow fragment of *E. coli* DNA polymerase I; ss, single-stranded; ds, double-stranded; sp, singly-primed; nt, nucleotide(s); BSA, bovine serum albumin; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, *p*-nitrotetrazolium-blue; SSE, sum of squares of errors; R^2, correlation coefficient.

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growing DNA chain (Lee & Hurwitz, 1990; Burgers, 1991; Tsurimoto & Stillman, 1991a; Lee et al., 1991a,b; Podust et al., 1992a). This complex prevents pol α from further elongation and favors pol δ to bind and start leading strand synthesis. Pol α/primase then starts synthesis on the lagging strand (Tsurimoto & Stillman, 1991b). This interplay between pol α and pol δ provides the coordinated DNA synthesis on both strands. Also on the lagging strand, completion of the Okazaki fragments is performed either by pol δ or by pol ϵ (Burgers, 1991). These results suggest the existence of a replication-competent (RC) complex in the cell, which, by analogy with the pol III holoenzyme of *E. coli*, should comprise pol α, pol δ, and/or pol ϵ and other auxiliary factors such as RF-C and PCNA.

Pol α has been purified by several eukaryotic sources as a four-subunit enzyme with associated DNA primase activity [reviewed in Wang (1991)], and it has been shown to be absolutely required for initiation of DNA replication both *in vitro* and *in vivo* (Johnson et al., 1985; Pizzagalli et al., 1988; Challberg & Kelly, 1989; Stillman, 1989). Pol α is likely to be part of a larger multiprotein complex, since several laboratories have isolated pol α as a larger complex containing a number of enzymatic activities such as pol, exonuclease, and ATPase (Hübscher et al., 1982; Baril et al., 1983; Pritchard & DePamphilis, 1983; Hübscher & Ottiger, 1984; Takada et al., 1986; Vishwanatha et al., 1986; Ottiger et al., 1987; Biswas & Biswas, 1988). In addition, a 21S enzyme complex has been isolated from HeLa cells which contains pol α, a DNase, DNA ligase, DNA topoisomerase I, RNase H, and PCNA and which is active in the SV40 *in vitro* DNA replication system (Malkas et al., 1990). These complexes, however, were labile and would explain why the isolation of a stable RC complex has proven to be so difficult.

In this paper, we present the isolation and characterization from calf thymus of a RC complex composed at least of pol α/primase, pol δ, and RF-C. This RC complex is functionally active in replication of natural templates, can coordinate primer synthesis and elongation by pol α/primase and pol δ, respectively, and forms an isolatable holoenzyme on spDNA in the presence of PCNA and ATP.

MATERIALS AND METHODS

Chemicals. [^{3}H] dTTP (40 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were from Amersham, and unlabeled dNTPs were from Boehringer. Whatman was the supplier of the GF/C and DE-81 filters. Immobilon-P nylon membrane was from Millipore. All other reagents were of analytical grade and purchased from Merck and Fluka.

Nucleic Acid Substrates. The homopolymer poly(dA)$_{400}$ (Pharmacia) was mixed (at weight ratios in nucleotides of 10:1) with the oligomer (dT)$_{12-18}$ (Pharmacia) in 20 mM Tris-HCl (pH 8.0), containing 20 mM KCl and 1 mM EDTA, heated at 65 °C for 5 min, and then slowly cooled at room temperature.

The ssDNA of M13 (mp11) was prepared as described (Sambrook et al., 1989). A 40-mer oligonucleotide primer complementary to nt 7041−7080 of the M13 genome was prepared as described (Podust & Hübscher, 1993). For the preparation of the spDNA, ssDNA (0.1 mg/mL) was mixed with the 40-mer oligonucleotide (2 μg/mL) in 10 mM Tris-HCl buffer, pH 7.8, 2.5 mM $MgCl_2$, and 0.125 M NaCl. The mixture was heated at 70 °C for 15 min with subsequent slow cooling at room temperature. The terminally mismatched poly(dA)/oligo(dT-[^{3}H]dCMP)$_{10:1}$ substrate for the exonuclease assay was prepared as described (Focher et al., 1989).

Enzymes and Proteins. Pol α, pol δ, and RF-C were purified from fetal calf thymus as described (Weiser et al., 1991; Podust et al., 1992a). One unit of pol activity corresponds to the incorporation of 1 nmol of total dTMP into acid-precipitable materials in 60 min at 37 °C in a standard assay containing 0.5 μg (nucleotides) of poly(dA)/oligo(dT)$_{1:10}$ and 20 μM dTTP. Human PCNA was overexpressed in *E. coli* strain BL21(DE3) harboring the expression plasmid pT7/PCNA and purified as described by Fien and Stillman (1992). Monoclonal antibodies against pol α, SJK 132-20 (Tanaka et al., 1982), were prepared as described (Weiser et al., 1991).

Buffers. Buffer A: 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 1 mM EDTA, 1 mM ATP, 1 mM $MgCl_2$, 20% sucrose (w/v), 1 μg/mL pepstatin, 1 μg/mL leupeptin, and 1 μg/mL aprotinin. Buffer B: 50 mM Tris-HCl, pH 7.5 (unless otherways stated), 5 mM DTT, 1 mM EDTA, 1 mM ATP, 1 mM $MgCl_2$, 20% glycerol (v/v), 50 mM NaCl, 1 μg/mL pepstatin, 1 μg/mL leupeptin, and 1 μg/mL aprotinin. Buffer C: 50 mM Tris-HCl, pH 7.5, 2 mM DTT, 10 mM $MgCl_2$, 0.2 mg/mL BSA, 1 mM ATP, and 50 mM NaCl. Buffer BDB: 50 mM Bis-Tris, pH 6.6, 1 mM DTT, 0.25 mg/mL BSA, and 6 mM $MgCl_2$. Buffer TDB: 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.2 mg/mL BSA, and 10 mM $MgCl_2$.

Enzymatic Assays. (i) RF-C-Independent Pol Assay. A final volume of 25 μL contained the following components: buffer BDB; 20 μM [^{3}H]dTTP (1.5 Ci/mmol); and 0.5 μg of poly(dA)/oligo(dT)$_{10:1}$. When indicated, 100 ng of PCNA was added together with the enzyme to be tested. All reactions were incubated for 15 min at 37 °C and precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined as described (Hübscher & Kornberg, 1979).

(ii) RF-C-Dependent Pol Assay. A final volume of 25 μL contained the following components: buffer TDB; 1 mM ATP; dATP, dGTP, and dCTP each at 50 μM; 20 μM [^{3}H]-dTTP (1.5 Ci/mmol); 100 ng of spDNA; 100 ng of PCNA; 500 ng of SSB; and enzyme to be tested. Reactions were incubated 30 min at 37 °C, and precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined as described (Hübscher & Kornberg, 1979). One unit of RF-C-dependent pol activity corresponds to the incorporation of 1 nmol of total dNMPs into acid-precipitable material in 60 min at 37 °C.

(iii) 3′→5′ Exonuclease Assay. A final volume of 25 μL contained the following components: buffer BDB; 0.5 μg of mismatched poly(dA)/oligo(dT-[^{3}H]dCMP); and enzyme to be tested. Reactions were performed as described (Focher et al., 1989). One unit of exonuclease activity is defined as the amount of enzyme which catalyzes the release of 1 nmol of mismatched dNMP at 37 °C in 1 h.

(iv) DNA Primase Assay. A final volume of 25 μL contained the following components: buffer TDB; GTP, CTP, and UTP each at 200 μM; 1 mM ATP; 100 ng of ssDNA; and enzyme to be tested. When indicated, 100 ng of PCNA and 500 ng of SSB, alone or in combination, were added together with the enzyme to be tested. Reactions were incubated 15 min at 37 °C; then 2 units of KF were added together with dATP, dGTP, and dCTP each at 50 μM, and

20 μM [^{3}H]dTTP (1.5 Ci/mmol), and incubation was continued for 5 min. Products were precipitated with 10% trichloroacetic acid, and the insoluble radioactive material was determined as described (Hübscher & Kornberg, 1979).

(v) Priming and Elongation Assay. A final volume of 25 μL contained the following components: buffer TDB; GTP, CTP, and UTP each at 200 μM; 1 mM ATP; 100 ng of ssDNA; and enzyme to be tested. The enzyme was preincubated 5 min at 37 °C in the reaction mixture in the absence or in the presence of dATP, dGTP, and dCTP, each at 50 μM, and 20 μM [^{3}H]dTTP (1.5 Ci/mmol), as indicated in the figure legends. For product analysis on the alkaline gel, dATP, dGTP, and dTTP, each at 100 μM, and 10 μM [α-^{32}P]-dCTP (25 Ci/mmol) were used. The incubation was then continued for the time indicated. Labeled dNTPs were supplemented where they were absent in the preincubation mixture; 100 ng of PCNA and 500 ng of SSB, either alone or in combination, were added to the mixture at the beginning of the reaction or after 5 min of preincubation, as indicated in the figure legends. Reactions were precipitated with 10% trichloroacetic acid, and the insoluble radioactive material was determined as described (Hübscher & Kornberg, 1979).

(vi) Other Assays. Topoisomerase I was assayed as described (Liu & Miller, 1981); DNA helicase activity was assayed as described (Thömmes & Hübscher, 1990); endonuclease and RNase H activities were assayed as described (Weiser et al., 1991).

Purification of the RC Complex from Calf Thymus. Fifty grams of calf thymus was resuspended in 3 volumes of buffer A and homogenized first in a Waring Blendor and then by 5−10 strokes in a Dounce homogenizer. The homogenate was centrifuged at 12000*g* for 30 min in a Sorvall GSA rotor and the supernatant collected (fraction I). Fraction I was loaded onto a 50 mL phosphocellulose column equilibrated in buffer A containing 50 mM NaCl. The column was washed with 4 volumes of the equilibration buffer and then eluted with a linear gradient from 50 mM to 600 mM NaCl in buffer A. A peak of pol activity which was RF-C-independent, but was stimulated by PCNA, eluted at 150 mM NaCl, followed by a second broad peak of RF-C-independent pol activity between 200 and 280 mM NaCl. The RF-C-dependent pol activity finally eluted as a single peak between 250 and 280 mM NaCl. The fractions containing the RF-C-dependent pol activity yielded fraction II.

Fraction II was dialyzed against buffer B and loaded onto a 20 mL sulfonyl-propyl−Sepharose column (Pharmacia) equilibrated in the same buffer. The column was washed with 5 volumes of the equilibration buffer and then first eluted with a gradient from pH 7.5 to pH 8.8 in buffer B. Under these conditions, the RF-C-independent pol activity eluted around pH 8.0, whereas the RF-C-dependent pol activity was retained on the column. A second linear gradient from 50 mM to 800 mM NaCl in buffer B, pH 8.8, eluted the RF-C-dependent pol activity as a single peak at 400 mM NaCl.

Active fractions were pooled (fraction III), dialyzed against buffer B, pH 8.5, and loaded onto a 10 mL DEAE column equilibrated in the same buffer. The column was washed with 5 volumes of the equilibration buffer and then eluted with a linear gradient from 50 mM to 500 mM NaCl. The RF-C-dependent pol activity eluted as a single peak at 300 mM NaCl. Fractions containing the RF-C-dependent pol activity were pooled to yield fraction IV.

Fraction IV was dialyzed against buffer B and gel-filtered through a 50 mL Sephacryl HRS-500 column (1.5 cm × 11.5 cm) equilibrated in buffer B containing 100 mM NaCl. The RF-C-dependent polymerase activity eluted as a single peak in the molecular weight range of 1×10^6.

Fractions active in the RF-C-dependent pol assay were pooled (fraction V), dialyzed against buffer B, and loaded onto a 5 mL phosphocellulose column equilibrated in buffer B. The column was washed with 5 volumes of the equilibration buffer and then eluted with a linear gradient from 50 mM to 600 mM NaCl in buffer B. The RF-C-dependent pol activity eluted as a single peak around 250 mM NaCl. The fractions containing the RF-C-dependent pol activity yielded fraction VI. They were stored at −80 °C and used for the enzymatic and immunological characterization.

For size-exclusion chromatography, 0.5 mL of fraction VI was loaded onto a 50 mL Sephacryl S-400 column (1.5 cm × 11.5 cm) equilibrated in buffer B containing 100 mM NaCl. The S-400 column was previously calibrated with aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa). Each calibration run was performed twice.

Immunoblot Analysis of the RC Complex. Polyclonal antibodies against mouse pol δ were prepared from rabbits as described (Cullmann et al., 1993). Antibody against the 40 kDa subunit of human RF-C was a gift of J. Hurwitz (Chen et al., 1992). Antibody against calf thymus pol α catalytic subunit was a gift of A. M. Holmes (Holmes et al., 1986). Monoclonal antibody against human pol ϵ catalytic subunit was a gift of J. Syväoja (Uitto et al., 1995). SDS−PAGE was as described (Laemmli, 1970). Proteins were transferred to an Immobilon-P nylon membrane by electroblotting with a Biorad Trans-blot apparatus, according to the manufacturer's protocol. The membrane was blocked with a 7% (w/v) solution of milk powder and then incubated in the presence of the appropriate antibody. Cross-reactivity of antibody to proteins was detected by utilizing alkaline phosphatase-coniugated secondary antibodies with NBT and BCIP as chromogenic substrates as described (Sambrook et al., 1989).

Immunoprecipitation of the RC Complex. Twenty micrograms (total protein) of fraction VI was incubated 10 min at 37 °C and then 12 h at 4 °C in buffer B without DTT, in the presence or in the absence of 20 μg of the monoclonal antibody SJK 132-20, specific for the catalytic subunit of pol α (Tanaka et al., 1982); 0.02 g of protein A−Sepharose (Pharmacia) swollen in buffer B was then added and the incubation continued for additional 4 h at 4 °C. The reaction mixture was then centrifuged for 10 min at 12000*g*, the supernatant was removed (fraction S), and the pellet (fraction P) was washed 3 times with buffer B containing 100 mM NaCl. After centrifugation, both fraction P and fraction S were heated for 10 min at 100 °C and subjected to SDS−PAGE. Western blotting and immunological detection of pol α, pol δ, and RF-C were performed as described above.

Isolation of the RC Complex Bound to a Primer-Template by Gel Filtration. A final volume of 100 μL contained buffer TDB, 10 mM $MgCl_2$, 400 ng of spDNA, 2 μg of SSB, and 1 mM ATP; 0.2 unit of the RC complex was incubated 3 min at 37 °C in the presence or in the absence of 500 ng of

PCNA, as indicated in the figure legend. The mixture was cooled on ice and immedately subjected to size-exclusion chromatography on a 1.8 mL Biogel A5m column (0.5 cm × 9 cm) equilibrated in buffer C. Fractions were tested for RF-C-dependent pol activity by incubating 44 μL together with 6 μL of a mixture which yielded dATP, dGTP, and dCTP, each at 50 μM; 20 μM [^{3}H]dTTP (1.5 Ci/mmol); 1 mM ATP; 1 μg of SSB; and when indicated 200 ng of PCNA, 200 ng of spDNA, and 0.2 unit of RC complex. Reactions were incubated for 30 min at 37 °C and precipitated with 10% trichloroacetic acid. Insoluble radioactive materials were determined as described (Hübscher & Kornberg, 1979).

Alkaline Gel Electrophoresis. Reactions were incubated under the conditions described for the priming and elongation assay and were digested by addition of 60 μg/mL proteinase K, 1% SDS, and 50 mM EDTA (final concentrations) for 30 min at 37 °C. Samples were subjected to phenol−chloroform extraction and precipitated with ethanol for 1 h at −80 °C. The DNA was resuspended in 50 mM NaOH and 1 mM EDTA, heated for 15 min at 60 °C, made to 3% ficoll (v/v), 0.03% bromophenol blue (w/v), and loaded onto a 1% agarose gel equilibrated in 30 mM NaOH, 1 mM EDTA, pH 8.0. The gel was run in the same buffer at 5 V/cm for 4 h at 4 °C, dried, and autoradiographed.

Steady-State Kinetic Measurements. A reaction mixture contained the following in a final volume of 25 μL: buffer BDB, 20 μM dTTP (1.5 Ci/mmol), RC complex, PCNA, ATP or ATPγ(S), and the template-primer, as indicated in the figure legends. All reactions were incubated at 37 °C for 5 min and precipitated with trichloroacetic acid, and the insoluble radioactive material was determined as described (Hübscher & Kornberg, 1979).

Steady-State Kinetic Data Analysis. K_m and V_{max} values were calculated according to the Michaelis−Menten equation. Analysis of the kinetic cooperativity and determination of the apparent dissociation constants K_1 and K_2 were performed according to the Adair's equation in the form:

$$v/V_{max} = \frac{K_2[S] + 2[S]^2}{2K_1K_2 + K_2[S] + [S]^2}$$

Hill numbers (n_H) were calculated according to the Hill equation in the form $v/V_{max} = [S]^n/(K + [S]^n)$ where $n = n_H$.

Fitting of the experimental data to the equations was performed by computer simulation using a least-squares curve-fitting method.

Protein and Nucleic Acids Determination. Protein concentrations were determined according to Bradford (1976). Poly(dA)$_{400}$ and oligo(dT)$_{12-18}$ concentrations were determined spectrophotometrically according to the manufacturer's protocol.

RESULTS

Specific Complementation Assay for Simultaneous Isolation of RF-C and a PCNA-Dependent Pol. In order to discriminate between the activity of free pols, RF-C, and a PCNA-dependent pol, two complementation assays were used (see also Materials and Methods): (i) the RF-C-independent assay which utilizes poly(dA)/oligo(dT) as the DNA template and enables us to discriminate between pol δ (which requires the addition of PCNA) and pol α or ϵ (which are PCNA-independent under these conditions). The activity detected by this assay was referred to as RF-C-independent pol activity. (ii) The RF-C-dependent assay, which utilizes SSB-covered spDNA as the template and is in the presence of PCNA and ATP. Under these conditions, pol δ and pol ϵ, but not pol α, are able to utilize the SSB-covered spDNA as template, but these enzymes absolutely require RF-C for their activity (Burgers, 1991; Tsurimoto & Stillman, 1991b; Lee et al., 1991a,b; Podust et al., 1992a). This assay enables detection of the simultaneous presence of pol δ and/or pol ϵ with RF-C in the fractions tested. The activity measured in this assay was therefore referred to as RF-C-dependent pol activity.

Table 1: Purification of a RC Complex from Calf Thymus

fraction	proteins (mg)	activity (units)[a]	specific activity (units/mg)	recovery (%)	purification (*x*-fold)
crude extract (fraction I)	955	95.5	0.1	100	1
phosphocellulose (fraction II)	17	96	5.6	101	55
SP-Sepharose (fraction III)	10	120	12	125	120
DEAE-cellulose (fraction IV)	4	100	25	105	250
HRS-500 (fraction V)	1.5	52	35	55	350
phosphocellulose (fraction VI)	0.4	40	100	42	1000

[a] One unit is defined as 1 nmol of dNTPs incorporated at 37 °C in 60 min in the RF-C-dependent assay, as described under Materials and Methods.

The RC Complex Contains Pol α*, Pol* δ*, and RF-C.* Table 1 summarizes the purification: the apparent increase in the recovery after the first steps is likely due to the removal of unspecific inhibitors. Figure 1A shows the analysis of the polypeptide composition of the purified RC complex (fraction VI) by SDS−PAGE. The identity of the catalytic subunit of pol α (p 180), pol δ (p 125), and one of the small subunits of RF-C (p 40) was unambiguously identified by immunoblot analysis (see below and Figure 1B). The other subunits were identified on the basis of the known polypeptide structure of these three enzymes: for pol α, the p 72 subunit and the two DNA primase subunits p 60 and p 50; for pol δ, the small subunit p 45; and for RF-C, the large subunit p 140 and two of the small subunits p 35 and p 33. In addition, a few other unknown bands were present in the fraction. Since further attempts to purify the complex resulted in loss of its integrity, it was not possible to determine if these polypeptides were actually part of the complex. The observed instability after a certain degree of purity could be due either to an excessive dilution or to the removal of some yet unidentified structural components, required for the integrity of the complex. In order to identify which enzymes were associated with the RF-C-dependent pol activity, fraction VI was tested with antibodies against mammalian pol α, pol δ, pol ϵ, and RF-C. As shown in Figure 1B, RF-C, pol δ, and pol α were all present in the fraction. No pol ϵ was detected. Fraction VI was further analyzed by size-exclusion chromatography on a Sephacryl S-400 column. As shown in Figure 2A, the RC complex activity eluted as a single peak, before the marker thyroglobulin (669 kDa), yielding a molecular mass of about 900 kDa. The peak fractions of the size-exclusion chromatography were also tested with the separate antibodies. As shown in Figure 2B, RF-C, pol α, and pol δ were present together in the same fraction.

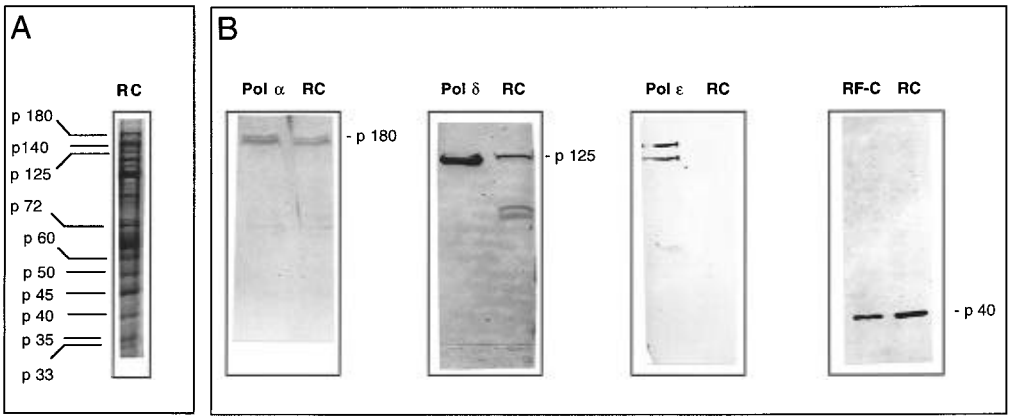

FIGURE 1: Polypeptide composition of the RC complex. Panel A: SDS−PAGE of fraction VI. Four micrograms (total proteins) was loaded onto a 7.5% polyacrylamide gel. Gel electrophoresis was performed as described under Materials and Methods. Panel B: Immunoblot analysis of fraction VI with antibodies against pol α, pol δ, pol ϵ, and RF-C. The antigens (either RC complex or purified calf thymus pol α, pol δ, pol ϵ, or RF-C) are indicated on the top of each lane. Gel electrophoresis, Western blot, and immunological analysis were performed as described under Materials and Methods.

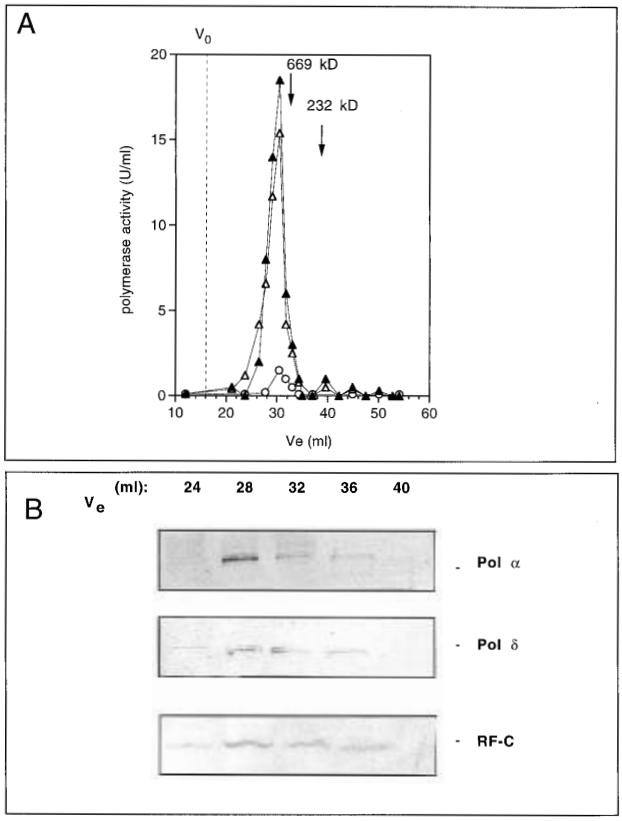

FIGURE 2: Size-exclusion chromatography of the RC complex on a Sephacryl S-400 column. Gel filtration was performed as described under Materials and Methods in the presence of 1 mM ATP. The elution points of catalase (232 kDa) and thyroglobulin (669 kDa) are indicated by arrows with the corresponding molecular masses. Panel A: Activity profile. Fractions were tested for activity as described under Materials and Methods; open triangles, RF-C-independent activity; filled triangles, RF-C-dependent activity. Panel B: Immunoblot analysis of the S-400 fractions with antibodies against pol α (first row), pol δ (second row), and RF-C (third row). The corresponding elution volume (V_e) in milliliters is indicated on the top of the figure. Gel electrophoresis and immunoblot analysis were performed as described under Materials and Methods.

ATP Is Required for the Stability of the RC Complex. As outlined under Materials and Methods, all buffers used during the purification included 1 mM ATP. This was found to be crucial for the stability of the RC complex, since omission of ATP at any step during the purification resulted in the loss of the RF-C-dependent pol activity. In Figure 3A, the elution profile of the same phosphocellulose fraction shown in Figure 2, purified through size-exclusion chromatography, but in the absence of ATP, is shown. Omission of ATP caused destabilization of the RC complex as reflected by the disappearance of the peak at 900 kDa with the corresponding loss of more than 80% of the RF-C-dependent pol activity. Instead, extra peaks appeared at lower molecular masses: (i) a peak of RF-C-independent pol activity which was partially stimulated by PCNA eluted at a molecular mass between 600 and 500 kDa; (ii) a peak of RF-C activity eluted at a molecular mass of about 300 kDa; (iii) a peak of pol δ activity eluted at a molecular mass of about 160 kDa. Immunoblot analysis of the fractions revealed that the peak at 500−600 kDa contained mainly pol α and pol δ, with some residual RF-C, and confirmed that the peak at 300 kDa contained only RF-C and the peak at 160 kDa only pol δ (Figure 3B). These results indicated that ATP was absolutely required for the stability of the complex since in its absence the RC complex apparently dissociated into different subassemblies.

Immunoprecipitation of the RC Complex with Monoclonal Antibodies against Pol α. The physical association of pol α, pol δ, and RF-C within the RC complex was confirmed by coimmunoprecipitation experiments. Monoclonal antibody SJK 132-20, specific for the catalytic subunit of pol α, were incubated together with fraction VI. The immunocomplexes formed were then precipitated by addition of protein A−Sepharose beads, followed by centrifugation. The presence of the protein of interest in both the pellet and the supernatant was then tested by immunoblot analysis. As shown in Figure 4, in the presence of the monoclonal antibody SJK 132-20, both pol δ and RF-C were coimmunoprecipitated with pol α and thus recovered in the pellet. The precipitation was absolutely dependent upon the formation of the immunocomplex between the antibodies and pol α, since in the absence of the antibodies none of the enzymes was retained in the pellet by the protein A−Sepharose beads. Some residual amounts of pol δ and RF-C were present in the supernatant even after incubation with the antibodies. This likely reflected the fraction of free enzymes present in the reaction mixture, after partial dissociation of the RC complex during the prolonged incubation.

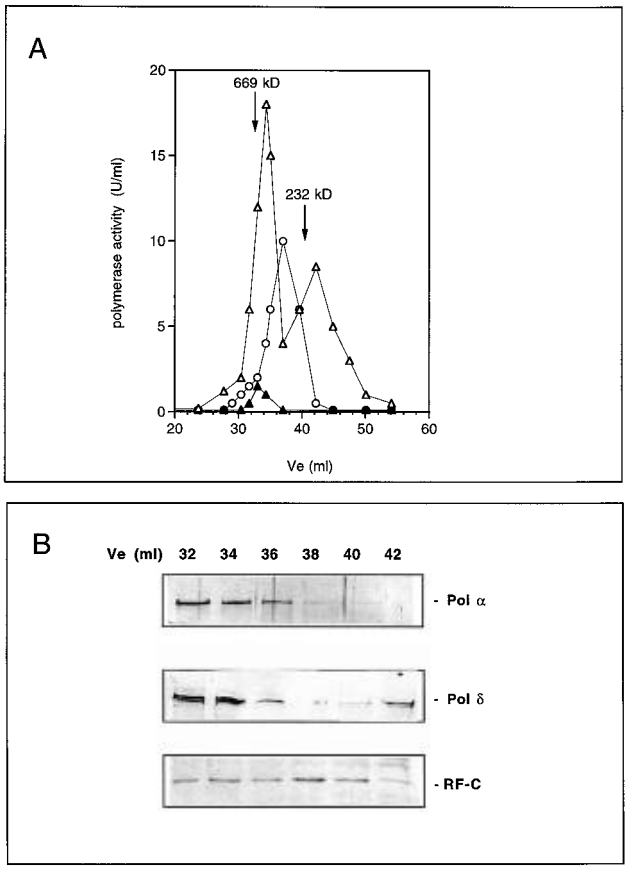

FIGURE 3: ATP is required for the stability of the RC complex. The size-exclusion chromatography of the RC complex was performed on a Sephacryl S-400 column as described under Materials and Methods, in the absence of 1 mM ATP. Panel A: activity profile; open triangles, fractions were assayed in the presence of poly(dA)/oligo(dT) as the DNA template and 100 ng of PCNA, under the conditions described under Materials and Methods for the RF-C-independent assay; filled triangles, fractions were tested in the presence of spM13DNA as the DNA template, *E. coli* SSB, PCNA, and ATP, under the conditions described under Materials and Methods for the RF-C-dependent assay; circles, fractions were tested in the presence of spM13DNA as the DNA template, *E. coli* SSB, PCNA, 1 mM ATP, and purified pol δ, under the conditions described under Materials and Methods for the RF-C-dependent assay. Panel B: Immunoblot analysis of the S-400 fractions with antibodies against pol α (first row), pol δ (second row), and RF-C (third row). The corresponding elution volume (V_e) in milliliters is indicated on the top of the figure. Gel electrophoresis and immunoblot analysis were performed as described under Materials and Methods.

The RC Complex Bound to a Primer-Template Can Be Isolated by Gel Filtration. The stability of the binding of the RC complex to a primer-template was tested by gel filtration through a Biogel A-5m column. The RC complex was incubated for 3 min at 37 °C with spDNA, SSB, and ATP in the presence or in the absence of PCNA and then gel-filtered at 4 °C. The fractions were tested for RF-C-dependent pol activity by complementation with the missing components. Under the conditions used, the spDNA elutes in the void volume, whereas free RC complex was in the included volume. As shown in Figure 5, when the RC complex was preincubated in the presence of PCNA, addition of only dNTPs was sufficient to detect the activity in the fractions containing the DNA template, indicating that the RC complex was bound to the primer-template. When PCNA was omitted from the preincubation mixture, however,

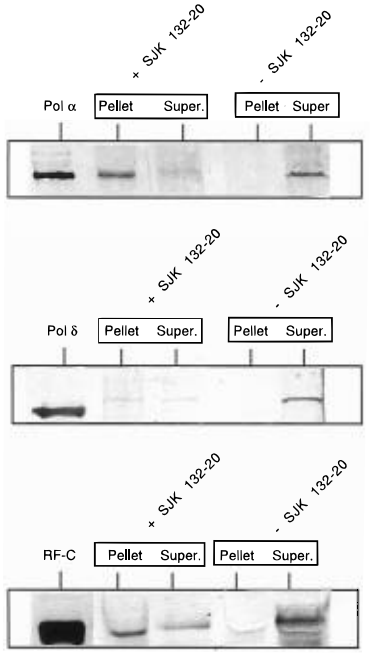

FIGURE 4: Immunoprecipitation of the RC complex with the monoclonal antibody SJK 132-20. Immunoprecipitation was performed as described under Materials and Methods. First row: immunoblot analysis of the pellet and supernatant with antibodies against pol α. Second row: immunoblot analysis of the pellet and supernatant with antibodies against pol δ. Third row: immunoblot analysis of the pellet and supernatant with antibodies against RF-C. Super.: supernatant.

only simultaneous addition of PCNA and the RC complex together with dNTPs restored the activity in the fractions containing the DNA, indicating that the RC complex was not bound to the primer-template. When the same fractions were tested by addition of spDNA, PCNA, and dNTPs, a peak of activity in the included volume showed that the RC complex was active, but failed to bind to the primer-template. These results indicated that PCNA was required in order to stabilize the RC complex to a primer-end. It has been shown that purified RF-C bound to a template-primer could be isolated by gel filtration in the absence of PCNA (Lee & Hurwitz, 1990). Thus, our results suggested that within the RC complex, RF-C required PCNA in order to promote a stable binding of the RC complex to a DNA primer.

Characterization of the Enzymatic Activities Associated with the RC Complex. The isolated RC complex was tested for the presence of a number of enzymatic activities. As summarized in Table 2, it contained, besides pol α, pol δ, and RF-C, only DNA primase and 3′→5′ exonuclease activities, the former associated with pol α and the latter with pol δ, respectively. These results indicated that both pols still retain these activities within the RC complex. Next, the activity of the RC complex on different DNA templates was compared with purified homologous pol α and pol δ (Table 3). With poly(dA)/oligo(dT) as the template and in the absence of PCNA, the RC complex showed almost the same activity as pol α, whereas pol δ was inactive. In the presence of PCNA, pol α was inhibited, whereas pol δ and the RC complex were both stimulated. With SSB-covered spDNA as the template and in the presence of ATP, pol α, pol δ, and the RC complex were totally inactive when PCNA was absent. When PCNA was added, pol δ and pol α were still inactive, whereas the RC complex became competent for DNA synthesis. Addition of RF-C did not influence the

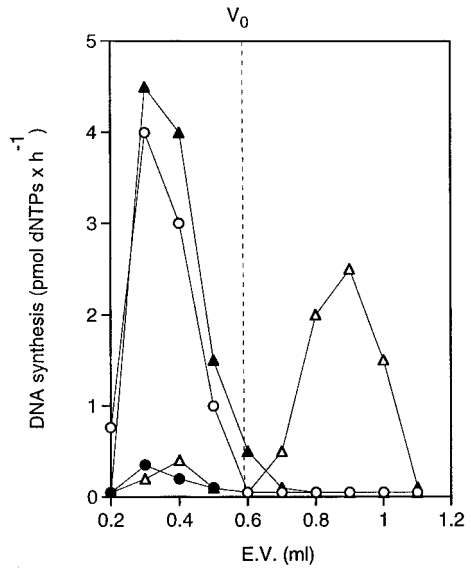

FIGURE 5: Isolation of the RC complex bound to spDNA by gel filtration. 0.2 unit of the RC complex was preincubated for 3 min at 37 °C under the conditions described under Materials and Methods. The reaction was then chilled on ice and chromatographed through a Biogel A-5m column at 4 °C. In the first experiment, the RC complex was preincubated together with spDNA in the presence of 500 ng of PCNA. After gel filtration, the fractions were tested for RF-C-dependent activity in the presence of dNTPs only (open circles). In the second experiment, the RC complex was preincubated together with spDNA in the absence of PCNA. After gel filtration, the fractions were tested for RF-C-dependent activity in the presence of (i) dNTPs and 200 ng of PCNA (filled circles); (ii) dNTPs, 200 ng of PCNA, and 0.2 unit of the RC complex (filled triangles); and (iii) dNTPs, 200 ng of PCNA, and 200 ng of spDNA (open triangles). SSB (1 μg) and ATP (1 mM) were included in all reactions.

Table 2: Activities Tested in the RC Complex

pol α	yes[a,b]
pol δ	yes[a,b]
pol ε	no[a,b]
poly(ADP-ribose) polymerase	no[b]
RF-C	yes[a,b]
RP-A	no[b]
PCNA	no[b]
DNA primase	yes[a]
3′→5′ exonuclease	yes[a]
topoisomerase I	no[a]
DNA helicase	no[a]
RNase H	no[a]
ss/ds DNA endonuclease	no[a]

[a] Tested by specific assays as described under Materials and Methods. [b] Tested by immunoblot analysis.

activity of the RC complex, but stimulated, as expected, pol δ. Pol α, on the other hand, was still inactive. When ssDNA without SSB was used as the template, the DNA synthesis became strictly dependent on primase activity. According to that, only pol α and the RC complex, but not pol δ were found to be active in the presence of rNTPs. When SSB was added, both pol α and the RC complex were inhibited, but if PCNA was added together with SSB, the RC complex was stimulated to the same level as seen on the spDNA template, whereas pol α was further inhibited. Next, the two known inhibitors of mammalian DNA polymerases, aphidicolin and BuPdGTP, were tested (Table 4). Both inhibitors completely suppressed the activity of pol α, whereas pol δ was only partially inhibited. The activity of the RC complex was completely inhibited by both compounds in the RF-C-independent assay without PCNA, as was pol α. When, however, PCNA was added, the RC complex showed a moderate sensitivity, similar to pol δ. In the RF-C-dependent assay, the RC complex again showed the same sensitivity as pol δ. Taken together, these results suggested that both pol α and pol δ were active in the RC complex and have template specificities and inhibitor sensitivities similar to those of the individually purified enzymes.

Characterization of the RF-C-Dependent Pol Activity. The mechanism by which RF-C enables pol δ to utilize spDNA as a template *in vitro* has been studied in detail (Lee & Hurwitz, 1990; Burgers, 1991; Tsurimoto & Stillman, 1991b; Lee et al., 1991a; Podust et al., 1992a). The reaction requires PCNA and ATP, which act as substrates for RF-C, as well as SSB which covers the ssDNA preventing unspecific binding of RF-C. In order to verify if these requirements were the same for the RC complex, PCNA, ATP, and SSB were titrated in the RF-C-dependent assay. As shown in Figure 6A, the reaction was completely dependent on the presence of both PCNA and ATP. Neither PCNA nor ATP alone was able to support RF-C-dependent DNA synthesis by the RC complex. The requirement for ATP as the phosphate donor was absolute, since none of the other ribonucletide triphosphates tested could substitute for it (Figure 6A). It is known that ATP hydrolysis is required for the interaction of RF-C with PCNA. When the nonhydrolyzable analogue ATPγ(S) was used with the RC complex instead of ATP, the reaction was completely inhibited. Next, ATP, PCNA, and SSB were titrated in the assay. The ATP and PCNA dependence of the reaction fitted well to hyperbolic saturation curves from which an $[S]_{0.5}$ of 290 (±10) μM for ATP and an $[S]_{0.5}$ of 0.5 (±0.1) μg/mL for PCNA were derived (Figure 6B,C). The dependence from SSB was not hyperbolic, according to the fact that SSB did not act as a substrate for the reaction, but followed a bell-shaped curve with a maximum at 500 ng of protein per 100 ng of spDNA (Figure 6D). Thus, the RF-C-dependent DNA synthesis of the RC complex showed the same requirements as the reaction reconstituted *in vitro* starting from purified RF-C and pol δ (Podust et al., 1992a).

Combined Activity of DNA Primase, Pol α, Pol δ, and RF-C on ssDNA. Coordination of the activities of DNA primase, pol α, pol δ, and RF-C within the RC complex was studied by using ssDNA as the template. In Figure 7A, the effects of SSB and PCNA on the kinetics of DNA synthesis on ssDNA catalyzed by purified pol α or the RC complex are shown. In this assay, both pol α and the RC complex were incubated 5 min in the presence of unlabeled rNTPs, labeled dNTPs, and the DNA template. This allowed primer synthesis by the DNA primase and partial elongation by pol α to occur. Then the incubation was continued for 15 min in the absence or in the presence of PCNA and SSB, and aliquots were taken at different time points. If the reaction proceeded in the absence of PCNA and SSB, no significant differences were seen between purified pol α and the RC complex. If SSB and PCNA were added after 5 min of incubation, however, pol α was inhibited, whereas the DNA synthesis by the RC complex was stimulated 3-fold. The nature of this effect was then investigated in more detail.

Figure 7B shows the influence of PCNA and SSB on the primer synthesis reaction catalyzed by the DNA primase activity associated either with purified pol α or with the RC

Table 3: Comparison of the RC Complex to Purified Pol α and Pol δ on Different Templates

	template							
	poly(dA)/oligo(dT)[a]		sp M13[b]			ssM13[c]		
enzyme	−PCNA	+PCNA	−PCNA	+PCNA	+PCNA/RF-C	+rNTPs	+rNTPs/SSBs	+rNTPs/SSBs/PCNA
pol α	100[d]	60	0.1	0.06	0.04	3.1	2	1.1
pol δ	0.1	145	0.05	0.04	25	0.05	0.05	0.05
RC complex	120	190	0.07	25	22	4.7	3.8	20

[a] Reactions were performed as described under Materials and Methods for the RF-C-independent assay. [b] Reactions were performed as described under Materials and Methods for the RF-C-dependent assay. [c] Reactions were performed as described under Materials and Methods for the primer elongation assay. [d] In order to compare the three different enzymes, the activites were expressed as a percentage relative to the one of pol α on poly(dA)/oligo(dT) without PCNA, which was taken as 100%.

Table 4: Comparison of the RC Complex to Purified Pol α and Pol δ on Different Inhibitors

			inhibition (%)[a]	
enzyme	inhibitor (concn, μM)	template	−PCNA	+PCNA
pol α	aphidicolin (20)	poly(dA)/oligo(dT)[b]	90	95
	BuPdGTP (10)	poly(dA)/oligo(dT)	87	90
pol δ	aphidicolin (20)	poly(dA)/oligo(dT)[b]	nd[d]	75
	BuPdGTP (10)	poly(dA)/oligo(dT)	nd	60
	aphidicolin (20)	spM13 (SSBs, RF-C, ATP)[c]	nd	70
	BuPdGTP (10)	spM13 (SSBs, RF-C, ATP)	nd	65
RC complex	aphidicolin (20)	poly(dA)/oligo(dT)[b]	88	68
	BuPdGTP (10)	poly(dA)/oligo(dT)	85	60
	aphidicolin (20)	spM13 (SSBs, ATP)[c]	nd	67
	BuPdGTP (10)	spM13 (SSBs, ATP)	nd	65

[a] Percentage of inhibition relative to the activity of control reactions without the inhibitors. [b] Reactions were performed as described under Materials and Methods for the RF-C-independent assay in the absence or in the presence of the indicated concentration of the inhibitors. [c] Reactions were performed as described under Materials and Methods for the RF-C-dependent assay, in the absence or in the presence of the indicated concentration of the inhibitors. [d] nd, not determined since no activity is measurable for pol δ without PCNA. The same is true also for the RC complex in the RF-C-dependent assay.

complex. In this assay, the RNA primers were first made by the primase in the presence of unlabeled rNTPs, and they were then elongated in the presence of labeled dNTPs, by a short 5 min pulse with a 100-fold excess of KF (in terms of units) over both pol α and the RC complex. Thus, under these conditions, the amount of DNA synthesis is solely related to primer synthesis by the primase. Since KF is affected neither by SSB nor by PCNA, any effect on the DNA synthesis level can be related to DNA primase. No differences could be detected between the primase activity associated with the RC complex and pol α. Both were inhibited at the same level by SSB and were unaffected by PCNA. This result suggested that the observed stimulation of the activity of the RC complex by PCNA and SSB (Figure 7A) is specific for the DNA synthesis step, exclusively.

Pol δ Can Substitute for Pol α in the RC Complex for the Primer Elongation Reaction through a PCNA/RF-C-Dependent Mechanism. In Figure 7C, the effects of PCNA and SSB on the priming and elongation reaction are shown. Either pol α or the RC complex was incubated 5 min in the presence of the DNA template, unlabeled rNTPs, and labeled dNTPs. Then PCNA and SSB were added either separately or in combination, and DNA synthesis was determined after an additional 15 min incubation. Pol α was inhibited by PCNA and SSB either in combination or alone. Moreover, when added separately, SSB and PCNA inhibited the activity of the RC complex in a similar way to pol α, whereas the combination of them resulted in a stimulation of the RC complex. These results suggested that in the presence of PCNA and SSB, the RF-C-dependent reaction took place, when pol δ, instead of pol α, was involved in the elongation of the primers.

Next, both pol α and the RC complex were incubated for 5 min in the presence of only rNTPs, in order to allow primer synthesis (Figure 7D). Then labeled dNTPs were added in the presence or in the absence of PCNA and SSB. In this case, no differences could be detected between the activities of pol α and the RC complex since both were inhibited by PCNA and SSB, either in combination or alone. This result confirmed the previous observation that pol α is inhibited by SSB and PCNA under these conditions and suggested that at least partial elongation of the RNA primers by the pol α activity associated with the RC complex was required for the subsequent PCNA-dependent elongation. Taken together, these results suggested that the primer elongation reaction occurred in two steps: the first step of elongation of the RNA primers was performed specifically by pol α, whereas pol δ could not substitute for it even in the presence of PCNA and SSB. However, when DNA primers were available, addition of PCNA and SSB made the RF-C-dependent reaction take place where pol δ substituted for pol α.

The Extent of Primer Elongation by the RC Complex Is Influenced by PCNA. Figure 8 shows the product analysis of the reaction catalyzed by the RC complex on ssDNA. When the RC complex was incubated for 5 min in the presence of labeled dNTPs and unlabeled rNTPs, more than 90% of the products were less than 0.4 kb in nucleotide length (lane 1). Omission of rNTPs resulted in a complete loss of DNA synthesis (lane 2), confirming that the nucleotide incorporation observed truly reflected primer elongation. When the incubation of the RC complex was extended for an additional 15 min under the same conditions, again almost only short products of less than 0.4 kb accumulated (lane 5). This indicated that the product length was not dependent on the incubation time and reflected a distributive mode of DNA synthesis. When PCNA and SSB were added at the beginning of the reaction (lane 3), the incorporation observed in 20 min of incubation was lower with respect to RC alone (lane 5). Since SSB and PCNA inhibited pol α activity, the synthesis of short products was almost completely abolished. On the other hand, longer products ranging from 0.7 to 2 kb as well as some full-length products of 7.5 kb appeared. This suggested that after a first elongation by pol α in a distributive mode, the DNA primers can be elongated up to full-length products by pol δ in the presence of PCNA. Addition of BuPdGTP at the beginning

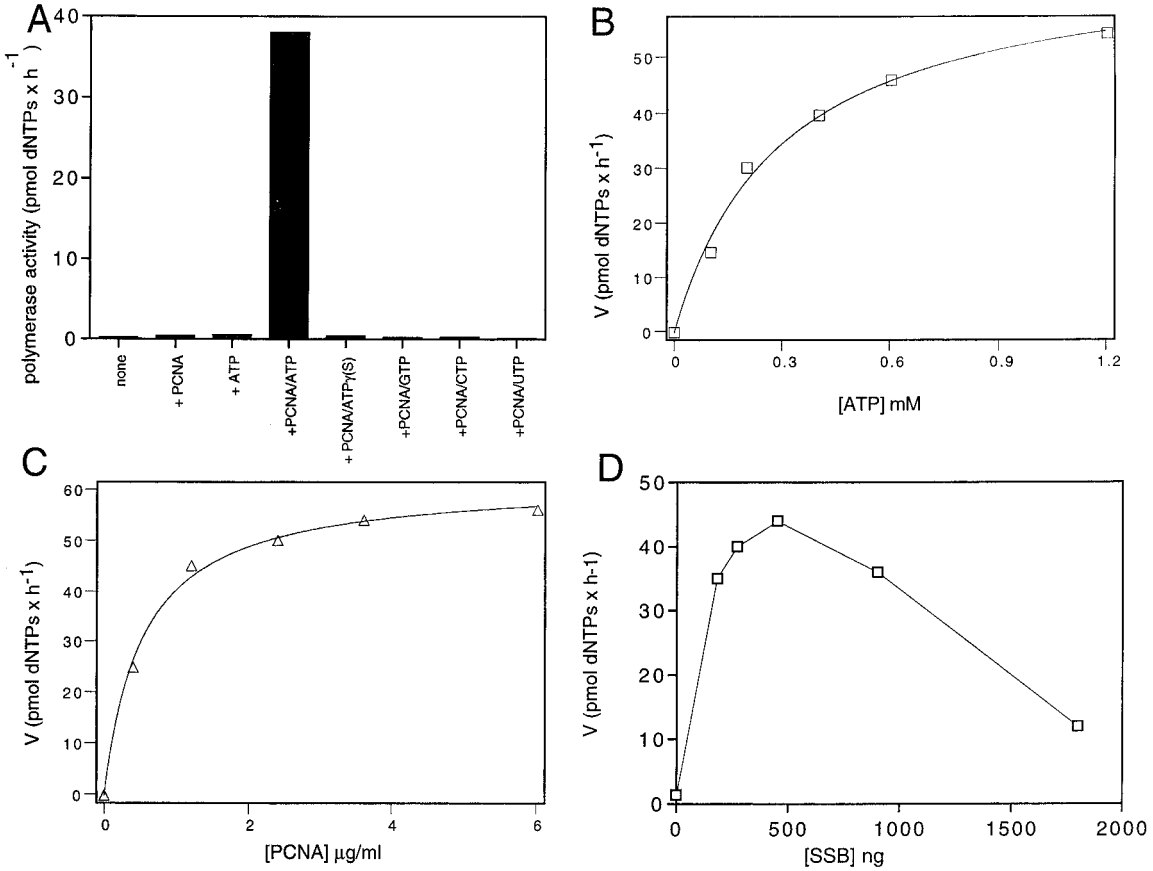

FIGURE 6: Characterization of the RF-C-dependent activity of the RC complex. The RF-C-dependent assay was performed as described under Materials and Methods. Panel A: Effect of PCNA and ATP. Fifty milliunits of RC complex, 500 ng of SSB, and 100 ng of spDNA were incubated in the assay mixture alone or in the presence of, respectively, (i) 100 ng of PCNA, (ii) 1 mM ATP, (iii) 100 ng of PCNA and 1 mM ATP, (iv) 100 ng of PCNA and 1 mM ATPγ(S), (v) 100 ng of PCNA and 1 mM GTP, (vi) 100 ng of PCNA and 1 mM CTP, (vii) 100 ng of PCNA and 1 mM UTP. Panel B: Titration of ATP in the RF-C-dependent assay. Fifty milliunits of the RC complex was incubated in the standard reaction mixture in the presence of different concentrations of ATP. Data were fitted to the hyperbolic equation: $v = V_{max}/(1 + S_{0.5}/[ATP])$. Panel C: Titration of PCNA in the RF-C-dependent assay. Fifty milliunits of RC complex was incubated in the standard reaction mixture in the presence of different amounts of PCNA. Data were fitted to the hyperbolic equation: $v = V_{max}/(1 + S_{0.5}/[PCNA])$. Panel D: Titration of SSB in the RF-C-dependent assay. Fifty milliunits of the RC complex was incubated in the standard reaction mixture in the absence or in the presence of increasing amounts of SSB.

of the reaction together with PCNA and SSB almost abolishes completely DNA synthesis (lane 4). This clearly indicated that the first step of elongation of the RNA primers was performed by pol α and it was required for the subsequent synthesis of full-length products by pol δ. When PCNA and SSB were added after 5 min of incubation and the reaction was then continued for an additional 15 min, long products between 0.7 and 7.5 kb accumulated (lane 6), indicating that the RC complex has switched from pol α to pol δ. If BuPdGTP was added together with PCNA and SSB after 5 min of incubation and then the reaction was continued for 15 min, only synthesis of short products (less than 0.4 kb) was completely suppressed, whereas longer products were still synthesized (compare lane 7 to lane 4). This experiment confirmed that the synthesis of full-length copies of the template was catalyzed by pol δ. Two families of products synthesized by pol δ can be distinguished (Figure 8, lane 6): one constituted by the full-length products; the second represented by many shorter products, clustered around 0.6−0.7 kb. This distribution can be explained in two ways: (i) the presence of pausing sites that cause dissociation of the polymerase or (ii) multiple priming events on the same template molecule. The latter case would restrict the synthesis performed by the RC complex to the gaps between adjacent primers, since the complex does not contain either RNase H or RNA/DNA helicase activity (Table 2), that could remove or displace the incoming RNA primer.

Kinetic Analysis of the Primer Binding Reaction Catalyzed by the RC Complex Supports the Hypothesis of an Asymmetric Pol Dimer. The kinetics of the primer binding reaction catalyzed by the RC complex were studied by using a steady-state kinetic approach. Given the presence of two pols in the RC complex (pol α and pol δ), the kinetics of the reaction should be different if these two enzymes acted independently or in coordination on the same substrate. Poly(dA)/oligo(dT) was used as the primer-template for this study since the activities of pol δ on this template depend on the presence of PCNA and thus can be easily discriminated from the PCNA-independent pol α. The values for the initial velocities which were used for calculation of the kinetic constants were determined as the amount of incorporated dTMP into the DNA template. In order to directly correlate these values to the variation of the DNA template concentration, the dTTP concentration was fixed in all the experiments. In preliminary experiments, we verified that the dependence of the initial velocity of the reaction from the dTTP concentration was unchanged under all the conditions used (data not shown). As shown in Figure 9A, in the absence of PCNA and ATP, the dependence of the reaction on the primer concentration followed normal hyperbolic kinetics,

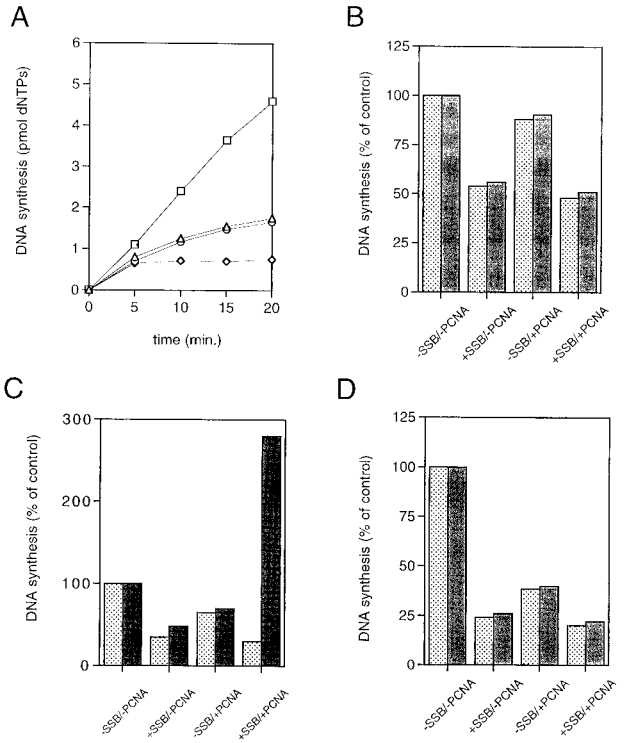

FIGURE 7: Coordinated activity of DNA primase, pol α, pol δ, and RF-C in the RC complex on ssDNA. Panel A: Kinetics of primer elongation by the RC complex or pol α on ssDNA in the absence or in the presence of PCNA and SSB. Twenty milliunits of the RC complex or 20 milliunits of pol α was preincubated 5 min at 37 °C in the presence of rNTPs and labeled dNTPs, under the conditions described under Materials and Methods for the priming and elongation reaction. The incubation was then continued in the absence or in the presence of 500 ng of SSB and 100 ng of PCNA. Aliquots were taken at different time points and processed as described. Triangles, activity of the RC complex in the absence of SSB and PCNA; squares, activity of the RC complex in the presence of SSB and PCNA; circles, activity of purified pol α in the absence of SSB and PCNA; rhombics, activity of purified pol α in the presence of SSB and PCNA. Panel B: Effect of SSB and PCNA on DNA primase activity associated with the RC complex or with purified pol α. Twenty milliunits of pol α (stippled boxes) or 20 milliunits of the RC complex (grey boxes) was incubated for 15 min at 37 °C under the conditions described under Materials and Methods for the DNA primase assay, in the absence or in the presence of 500 ng of SSB and 100 ng of PCNA either alone or in combination. Panel C: Effect of SSB and PCNA on the priming and elongation reaction catalyzed by the RC complex or pol α on ssDNA. Twenty milliunits of purified pol α (stippled boxes) or 20 milliunits of RC complex (grey boxes) was preincubated 5 min at 37 °C in the presence of rNTPs and labeled dNTPs under the conditions described under Materials and Methods for the priming and elongation reaction. The incubation was then continued for 15 min in the absence or in the presence of 500 ng of SSB and 100 ng of PCNA, either alone or in combination. Panel D: Effect of SSB and PCNA on the initial step of primer elongation by the RC complex or pol α on ssDNA. Twenty milliunits of pol α (stippled boxes) or 20 milliunits of the RC complex (grey boxes) was incubated 5 min at 37 °C in the presence of rNTPs, under the conditions described under Materials and Methods for the priming and elongation reaction. The reaction was then continued for 15 min by addition of labeled dNTPs in the absence or in the presence of 500 ng of SSB and 100 ng of PCNA, either alone or in combination.

according to a simple Michaelis−Menten mechanism. Under these conditions, the DNA synthetic activity was completely suppressed by BuPdGTP (Table 4). This suggested that only pol α was active. Thus, the calculated K_m for the substrate of 12 (±2) nM (Table 5) should reflect the affinity of the

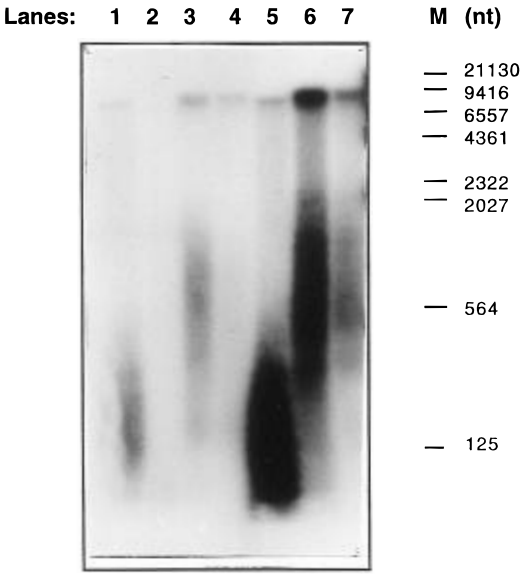

FIGURE 8: Analysis of the reaction products synthesized by the RC complex on ssDNA. Alkaline agarose gel electrophoresis was performed as described under Materials and Methods. Fifty milliunits of the RC complex was incubated at 37 °C under the conditions described under Materials and Methods for the priming and elongation reaction. Lane 1, the RC complex was incubated for 5 min at 37 °C in the presence of rNTPs and labeled dNTPs. Lane 2, the RC complex was incubated for 20 min at 37 °C in the presence of labeled dNTPs but without rNTPs. Lane 3, the RC complex was incubated 20 min at 37 °C in the presence of rNTPs, labeled dNTPs, 100 ng of PCNA, and 500 ng of SSB. Lane 4, as in lane 3, but with the addition of 0.1 mM BuPdGTP. Lane 5, as lane 1, but the reaction was incubated 20 min at 37 °C. Lane 6, as lane 5, but 100 ng of PCNA and 500 ng of SSB were added after the first 5 min of incubation. Lane 7, as lane 6, but 0.1 mM BuPdGTP was added together with PCNA and SSB. As molecular size marker, λ DNA was digested with *Hin*dIII and 5′-labeled with polynucleotide kinase and [γ-^{32}P]ATP. The position of the restriction fragments and the relative size in nt are indicated.

pol α active site for the 3′-OH primer. When both PCNA and ATP were added, the reaction showed a sigmoidal dependence on substrate concentration (Figure 9B). From the Hill equation, an apparent Hill number (n_H) of 1.8 was derived, suggesting that two active sites were involved in the reaction. In order to explain the observed kinetic positive cooperativity, we tested our experimental data according to different models by computer simulation. The Adair's equation for a dimeric enzyme with two nonidentical binding sites gave the best fit. According to this model, the apparent dissociation constants for the two sites (K_1 and K_2) were 67 (±5) nM and 10 (±3) nM, respectively (Table 5). Thus, the apparent positive cooperativity arises from the fact that the first site occupied by the substrate has lower affinity than the second. Comparison of the K_2 value with the K_m obtained in the reaction without PCNA and ATP suggested that under these conditions pol δ binds first, followed by pol α. If the binding reaction was driven exclusively by the relative affinities of both sites for the substrate, one would expect to observe negative cooperativity, the site with higher affinity being saturated first. When similar experiments were performed with purified pol α and pol δ, we were not able to reproduce the positive cooperativity observed with the RC complex (data not shown). The sigmoidal curve observed in the case of the RC complex, therefore, reflected the existence of some property of the complex that influenced the reaction.

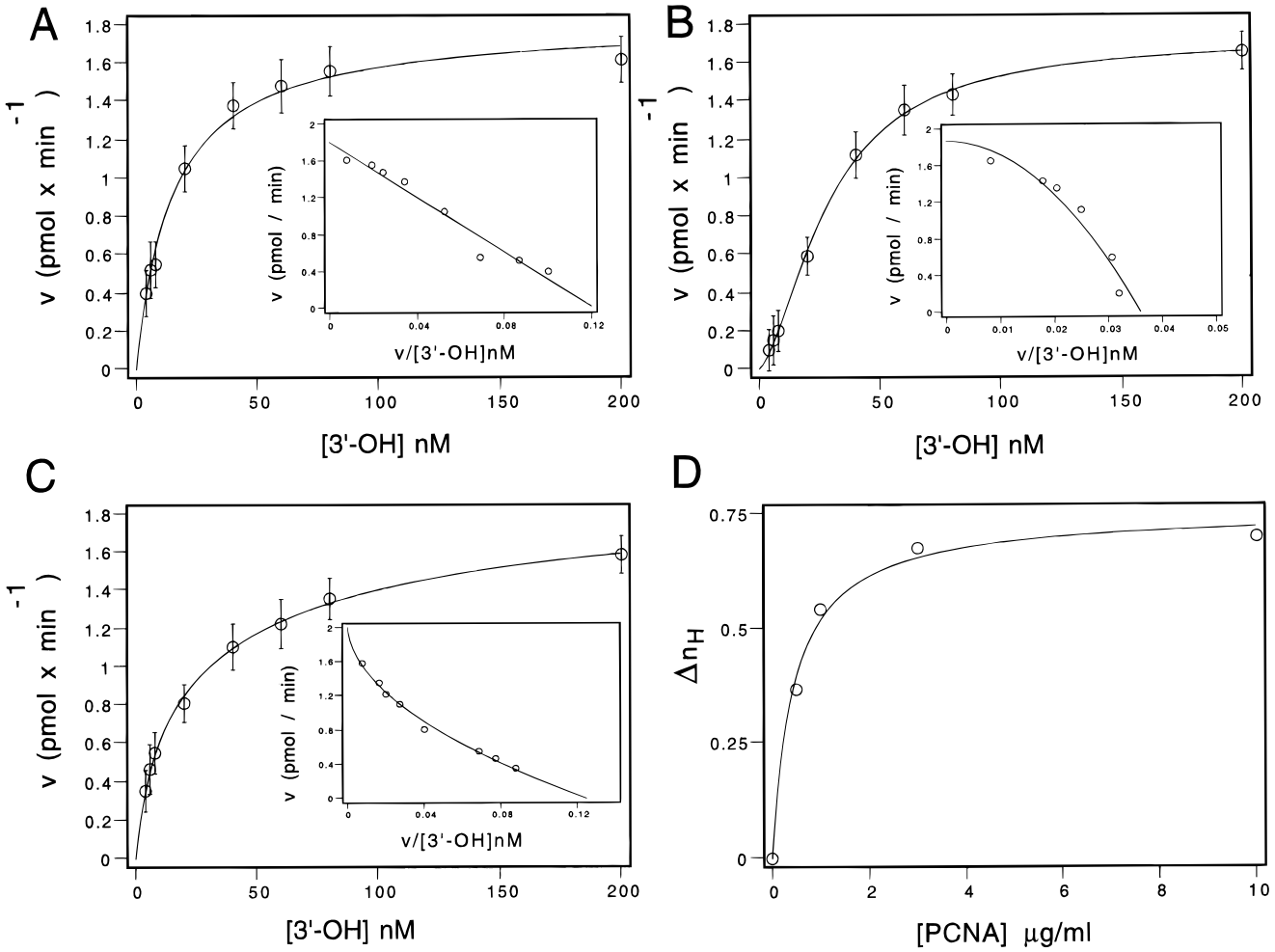

FIGURE 9: Kinetic analysis of the primer binding reaction catalyzed by the RC complex. Reactions were carried out in the presence of 50 milliunits of RC complex as described under Materials and Methods. The solid lines represent the best fit of the data points to the different rate equations obtained by computer simulation. Primer concentrations used in all the experiments were 4, 6, 8, 20, 40, 60, 80, and 200 nM 3′-OH ends, respectively. Panel A: Reactions were carried out in the presence of increasing concentrations of poly(dA)/oligo(dT) as the DNA template and in the absence of ATP and PCNA. SSE of the fitting, 0.008; R^2, 0.945. Panel B: Effect of ATP and PCNA on the primer binding reaction. Reactions were carried out in the presence of increasing concentrations of poly(dA)/oligo(dT) as the DNA template and in the presence of 100 ng of PCNA and 1 mM ATP. SSE of the fitting, 0.003; R^2, 0.999. Panel C: Effect of PCNA on the primer binding reaction. Reactions were carried out in the presence of increasing concentrations of poly(dA)/oligo(dT) as the DNA template and in the presence of 100 ng of PCNA without ATP. SSE of fitting, 0.002; R^2, 0.997. Insets: Eadie−Hoffstee plots of the same data. Data were fitted to the equation: $v = V_m - (Kv/[S]^n)$, were $n = n_H$. Panel D: Dependence of the increase in cooperativity of the reaction by the PCNA concentration. The apparent Hill number (n_H) was determined in a set of experiments by varying the concentrations of poly(dA)/oligo(dT) in the presence of 1 mM ATP in combination with different fixed concentrations of PCNA for each experiment. The increase in cooperativity was calculated as the difference between the n_H values determined for each PCNA concentration and the value in the absence of PCNA (n_{H0}, which was 1.012 ± 0.002). These differences (Δn_H) were then plotted against the PCNA concentrations. Data points were fitted to the hyperbolic equation: $\Delta n_H = \Delta_{max}/(1 + S_{0.5}/[PCNA])$, where Δ_{max} is the maximal increase and $S_{0.5}$ is the PCNA concentration at which $\Delta n_H = 0.5(\Delta_{max})$.

RF-C and PCNA Are Responsible for the Coordination between the Pol α and Pol δ Activities in the RC Complex. In order to investigate the apparent positive cooperativity observed for the primer binding reaction catalyzed by the RC complex, the effects of PCNA and ATP on the reaction were studied separately. As shown in Figure 9C, when only PCNA was added, the kinetics were again nonlinear, but showing negative cooperativity, with an apparent Hill number of 0.55. Since from the inhibition experiments it was suggested that in the presence of PCNA both pol α and pol δ were active, this result suggested that they acted independently on the substrate. The derived K_1 and K_2 values from the Adair's equation were 9 (±2) nM and 70 (±6) nM, respectively (Table 5), suggesting that the same active sites were responsible for the negative and positive cooperativity.

The previous results indicated that both PCNA and ATP were required in order to have positive cooperativity. Indeed, when ATP alone was added, neither positive, nor negative cooperativity was observed (data not shown). However, ATP hydrolysis is absolutely required together with PCNA in order to observe a positive cooperativity: when the nonhydrolyzable analogue ATPγ(S) was added in place of ATP together with PCNA, again a negative cooperativity was observed, suggesting that under these conditions pol α and pol δ activities were independent (data not shown). K_1 and K_2 values were 11 (±2) nM and 60 (±10) nM, respectively (Table 5). Finally, the effect of increasing amounts of PCNA on the degree of positive cooperativity shown by the RC complex in this system was studied. In Figure 9D, the dependence of the increase in the apparent n_H value of the

Table 5: Effect of PCNA, ATP, and ATPγ(S) on the Kinetic Parameters for the Primer Binding Reaction of the RC Complex

	none	+PCNA/ −ATP[a]	+PCNA/ +ATP	+PCNA/ +ATPγ(S)
K_m[b] (nM)	12	*c*		
K_1 (nM)	*d*	9	67	11
K_2 (nM)		70	10	60
n_H	1.01	0.55	1.8	0.6

[a] 100 ng of PCNA and 1 mM ATP or ATPγ(S) were added when indicated. [b] K_m, apparent Michaelis−Menten constant of the RC complex for the 3′-OH with poly(dA)/oligo(dT)$_{10:1}$ as the DNA substrate; K_1, K_2, apparent dissociation constants, respectively, for the first and second molecule of 3′-OH bound by the RC-complex with poly(dA)/oligo(dT)$_{10:1}$ as the DNA substrate, as derived by Adair's equation; n_H, apparent Hill number for the binding of the 3′-OH to the RC-complex with poly(dA)/oligo(dT)$_{10:1}$ as the DNA substrate, as derived by the Hill equation. Determination of the kinetic constants was performed as described under Materials and Methods. [c] In the case of either positive or negative cooperativity, K_m was not determined, since the reaction did not follow simple Michaelis−Menten kinetics. [d] In the absence of cooperativity, with one single active site involved in the reaction, $n_H = 1$ and the Adair's equation reduces to the simple Michaelis−Menten mechanism.

reaction on the PCNA concentration is shown. The data points fitted well to a hyperbolic equation, according to the hypothesis that this effect is triggered by an enzymatic reaction. Remarkably, the $[S]_{0.5}$ value for PCNA in this reaction was 0.45 (±0.05) μg/mL, almost identical to the value derived for the stimulation of the RF-C-dependent DNA synthesis (compare Figure 6C to Figure 9D). The fact that positive cooperativity was observed only in the simultaneous presence of PCNA and ATP, but not ATP(γ)S, as for the RF-C-dependent DNA synthesis (Figure 6A), pointed to a role for RF-C in mediating this effect.

DISCUSSION

In this work, we showed that calf thymus pol α/primase, pol δ, and RF-C form an isolatable replication competent (RC) complex. This conclusion was based upon the following observations:

(i) Copurification of pol α/primase, pol δ, and RF-C through five distinct chromatographic steps, including both ion-exchange and gel-filtration columns (Table 1). The presence of these activities during the purification has been monitored by using a specific complementation assay with an SSB-covered spDNA template as well as immunoblot analysis with specific antibodies (Figure 1).

(ii) Coelution of pol α/primase, pol δ, and RF-C through an S-400 gel filtration column, in the presence of ATP (Figure 2). Indeed, ATP was found to be essential for the stability of the RC complex (Figure 3).

(iii) Coimmunoprecipitation of pol δ and RF-C together with pol α by the monoclonal antibody SJK 132-20, specific for the pol α catalytic subunit. This indicated that these enzymes were physically associated in the RC complex (Figure 4).

(iv) Isolation of the RC complex bound to a primer-template after gel filtration through a Biogel A5m column, in the presence of PCNA (Figure 5). The fact that the RC complex could not be isolated as a stable holoenzyme with spDNA in the absence of PCNA, contrary to purified RF-C (Lee & Hurwitz, 1990), indicated that RF-C in the complex was required not only for loading PCNA on the template but also for maintaining the integrity of the RC complex as well as for the proper assembly of the RC complex onto a primer.

The complex was labile after a certain degree of purity, which might be due either to an excessive dilution or to the removal of yet unknown factors required for its integrity. Among a number of activities tested, the RC complex contained, beside pol α, pol δ, and RF-C, only DNA primase and 3′→5′ exonuclease activities (Table 2). Pol α, pol δ, and RF-C inside the RC complex showed the same template specificities and inhibitor sensitivity of the single purified enzymes from the same tissue (Tables 3 and 4). The activity of the RC complex in the RF-C-dependent pol assay showed the same dependence from ATP and PCNA as the one reconstituted *in vitro* starting from purified RF-C and pol δ (Figure 6).

RF-C seemed to coordinate the activity of pol α and pol δ in the reaction catalyzed by the RC complex on ssDNA. The RC complex was able to synthesize RNA primers, through the associated primase activity. These primers were then first specifically elongated by pol α. Then, addition of PCNA and ATP apparently resulted in a "switching" between pol α and pol δ, which then preferentially extended the primers in place of pol α. Evidence for this switching came from the following observations:

(i) Addition of SSB or PCNA alone suppressed synthesis by the RC complex on ssM13DNA, similarly to purified pol α (Figure 7C). The activity of the RC complex, but not of pol α, could be restored only by the simultaneous addition of SSB, PCNA, and ATP (Figure 7A,C), but only after pol α partially elongated the RNA primers (Figure 7D).

(ii) Alkaline gel analysis of the products of the reaction catalyzed by the RC complex on ssDNA showed that in the absence of PCNA only short fragments of less than 0.4 kb accumulated (Figure 8, lane 5). Upon addition of PCNA, products ranging from 0.7 kb up to full-length products of 7.5 kb were evident (Figure 8, lane 6). The synthesis of short products could be completely inhibited by BUPdGTP, whereas the synthesis of full-length products was only partially affected (Figure 8, lanes 4 and 7).

(iii) Kinetic analysis of the primer binding reaction catalyzed by the RC complex again suggested that "polymerase switching" occurred (Figure 9). In the presence of both PCNA and ATP, the reaction showed a positive cooperativity for primer binding, with an n_H of 1.8. Omission of ATP or its substitution with the nonhydrolyzable analogue ATPγ(S) in the presence of PCNA resulted in negative cooperativity. The best fit to these data was obtained through computer simulation according to the Adair's model for a dimeric enzyme with two nonidentical active sites. This analysis showed that the intrinsic dissociation constants for the 3′-OH primer of both pol α and pol δ are not changed by PCNA and/or ATP. The cooperativity, either positive or negative, depended on the order of binding of the substrate to these active sites. Comparison between the values derived from the Adair's equation for the binding of the first and second molecules of substrate in the different cases (Table 5) indicated that if ATP and PCNA were present pol δ bound first. This effect required ATP hydrolysis and was dependent upon the PCNA concentration, following a hyperbolic relationship superimposable to the one obtained for the RF-C-dependent pol activity (compare Figure 6C to Figure 9D). Thus, the interaction of pol δ in the RC complex with PCNA,

mediated by RF-C through an ATP-dependent mechanism, appeared to enable pol δ to substitute for pol α in primer elongation. When similar experiments were performed with purified pol α and pol δ, positive cooperativity was not observed, as expected in the case of two enzymes acting independently (data not shown). These results indicated that the observed positive cooperativity was a property of the RC complex and suggested that pol α and pol δ form an asymmetric pol dimer together with RF-C.

Reconstitution experiments *in vitro* with purified components suggested that the polymerase switching mechanism could play a role in the DNA replication in eukaryotes (Tsurimoto & Stillman, 1991). The properties of the RC complex described in this paper would also fit in this model. At an early stage of DNA replication, duplex DNA is opened at the origin (probably by an initiation complex), thus exposing a ss region. The RC complex composed of pol α, pol δ, and RF-C could then be recruited to an opened origin. Once properly positioned to the origin, the primase activity associated with the RC complex starts the synthesis of the first RNA primer on the leading strand. This primer is then taken up and partially elongated by pol α. It has been shown that this switching by primase and pol α occurs via an intramolecular mechanism (Sheaff et al., 1994). Thus, the primer end is not exposed outside the active site of pol α until the enzyme dissociates after the synthesis of a short stretch of DNA, due to its low intrinsic processivity (Matsumoto et al., 1990). The primer end is then available for binding, and two reactions could take place: (i) reinitiation by pol α on the same primer or (ii) switch to the pol δ active site. The interaction of PCNA with pol δ in the RC complex, mediated by RF-C in an ATP-dependent reaction, prevents reinitiation by pol α and favors binding of pol δ to the primer. The observation that PCNA and ATP suppressed the synthesis by pol α in the RC complex on a natural DNA template supports this hypothesis (Figure 7). When pol δ is engaged in processive synthesis on the leading strand, the primase could bind the ss region of the lagging strand and synthesize a new primer.

Studies with purified pol δ, RF-C, and PCNA (Burgers, 1991) showed that the rate of DNA synthesis by the pol δ holoenzyme on spM13DNA was about 50−100 nt s^{-1}. From Figure 8, lane 6, the appearance of full-length products after 20 min of incubation suggested that the rate of DNA synthesis on ssM13DNA by the RC complex was about 6.5 nt s^{-1}. RC complex activity on ssM13DNA was dependent on the DNA primase for RNA primer synthesis. On the contrary, in the above cited study with the purified pol δ holoenzyme, primers were already annealed to the template. Thus, the lower rate observed for the RC complex can be explained either with a lower primer concentration or if the priming step itself was rate-limiting for the reaction. The SV40 *in vitro* DNA replication system, reconstituted from purified components, also relies on the DNA primase activity for RNA primer synthesis. The incorporation rate measured in the reconstituted reaction has been reported to be about 6 nt s^{-1} (Ishimi et al., 1991), thus very similar to the one measured with the RC complex.

The isolation of a 21S enzyme complex from HeLa cells has been reported. This complex is able to support *in vitro* SV40 DNA replication (Malkas et al., 1990). It contained pol α as well as a number of other enzymatic activites such as an unidentified DNase, DNA ligase, DNA topoisomerase I, RNase H, and PCNA. In contrast, the RC complex from calf thymus described in this paper contained exclusively, among the various activities tested, two pols, namely, pol α with the associated DNA primase and pol δ with the intrinsic 3′→5′ proofreading activity and the auxiliary factor RF-C (Table 2).

It has been shown that pol α alone can fulfill the requirements for DNA synthesis in the *in vitro* SV40 DNA replication system, under the conditions known as the monopolymerase reaction (Wobbe et al., 1987; Eki et al., 1992). However, from reconstitution experiments, it has been demonstrated that pol δ and RF-C together with pol α are essential factors for *in vitro* SV40 DNA replication in the dipolymerase reaction (Lee et al., 1989; Eki et al., 1992). Indeed, in the current model based on the reconstituted *in vitro* SV40 DNA replication system, DNA replication in eukaryotic cells is thought to involve the coordinated action of pol α, pol δ, and RF-C (Waga & Stillman, 1994).

The differences in both molecular mass and composition between the 21S complex from HeLa cells and the RC complex from calf thymus could be due to differences in the isolation procedure. In this regard, it must be noted that, contrary to Malkas and colleagues, we also used gel filtration in the presence of ATP for the isolation of the RC complex. This is a nonequilibrium technique, which preserves only strong interactions. It is also possible that species- or cell type-specificity could account for the observed differences.

In eukaryotic cells, a third pol, pol ϵ, is thought to participate in DNA replication together with pol α and pol δ [reviewed in Hübscher and Spadari (1994) and Stillman (1994)]. Pol ϵ also interacts with PCNA and RF-C (Burgers, 1991; Lee et al., 1991b; Podust et al., 1992a; Maga & Hübscher, 1995). Genetic studies showed that the pol ϵ gene is essential for viability in yeast (Morrison et al., 1990), and more recent results seem to indicate that this enzyme actually links the DNA replication machinery to the S-phase checkpoint (Navas et al., 1995). On the other hand, a role for pol ϵ in the *in vitro* SV40 replication system has not been described so far (Waga et al., 1994; Waga & Stillman, 1994). It has been proposed that only pol α and pol δ participate in DNA replication, whereas pol ϵ is mainly involved in DNA repair (Nishida et al., 1988). The fact that we did not find pol ϵ associated with the RC complex does not exclude a role of this pol in DNA replication. One possible function for pol ϵ at the replication fork could be to complete the synthesis of the Okazaki fragments on the lagging strand (Burgers, 1991; Podust et al., 1993). It is possible that such an interaction requires additional factors that are missing in our preparation. Finally, pol ϵ could be involved in postreplicational repair, thus without interacting directly with the RC complex described in this paper.

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