Nucleic Acids Research, Vol. 20, No. 16 ***4159–4165***

Calf thymus RF-C as an essential component for DNA polymerase δ and ε holoenzymes function

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Received June 8, 1992; Revised and Accepted July 21, 1992

ABSTRACT

By using a complementation assay that enabled DNA polymerase δ and DNA polymerase ε to replicate a singly-DNA primed M13 DNA in the presence of proliferating cell nuclear antigen (PCNA) and *Escherichia coli* single-stranded DNA binding protein (SSB), we have purified from calf thymus in a five step procedure a multipolypeptide complex with molecular masses of polypeptides of 155, 70, 60, 58, 39 (doublet), 38 (doublet) and 36 kDa. The protein is very likely replication factor C (Tsurimoto, T. and Stillman, B. (1989) *Mol. Cell. Biol.* 9, 609 – 619). This conclusion is based on biochemical and physicochemical data and the finding that it contains a DNA stimulated ATPase which is under certain conditions stimulated by PCNA. Together RF-C, PCNA and ATP convert DNA polymerases δ and ε to holoenzyme forms, which were able to replicate efficiently SSB-covered singly-DNA primed M13 DNA. Calf thymus RF-C could form a primer recognition complex on a 3′-OH primer terminus in the presence of calf thymus PCNA and ATP. Holoenzyme complexes of DNA polymerase δ and ε could be isolated suggesting that these enzymes directly interact with the auxiliary proteins in a similar way. Under optimal replication conditions on singly-DNA primed M13 DNA the DNA synthesis rate of DNA polymerase δ was higher than of DNA polymerase ε. Based on these functional date possible roles of these two DNA polymerases in eukaryotic DNA replication are discussed.

INTRODUCTION

In eukaryotes recent data suggested that three different DNA polymerases (pol), called α, δ and ε, appear to be involved in DNA replication (reviewed e.g. [1–5]). Tsurimoto et al. [6] suggested that replication of SV40 DNA requires two pols, namely pol α/primase and pol δ. In addition at least three pol auxiliary proteins called proliferating cell nuclear antigen (PCNA) [7], replication protein A (RP-A) [8] (also called replication factor A [9] or single-strand binding protein (SSB) [10]) and replication factor C (RF-C) [11] are required. First, unwinding of the origin of replication occurs by the combined action of T antigen and RP-A, and then pol α/primase initiates DNA synthesis at the origin. After synthesis of relatively short stretches of DNA the two pol auxiliary proteins PCNA and RF-C come into action by forming a tight complex in the presence of ATP at the 3′-OH end of the growing DNA chain. The binding of RF-C and PCNA prevents pol α/primase from further binding to the 3′-OH end and instead favours pol δ to bind and start leading strand DNA synthesis. The dissociated pol α/primase cycles to the next priming site on the lagging strand to resume discontinuous replication. Simultaneously, pol δ proceedes to synthesize the leading strand continuously. This interplay of pol α and δ provides the coordinated DNA synthesis in both directions ([6] and Fig. 5 therein).

How does pol ε the third essential pol [12,13] come into play? Pol auxiliary proteins RP-A, PCNA and RF-C are required by pol δ for replication of natural DNA [14,15]. These three proteins also appear to interact with pol ε, but in a more complex way [16,17]. DNA replicaton by pol ε becomes resistant to salt upon addition of RF-C to a reaction mixture containing pol ε, PCNA and RP-A. The high degree of functional similarities between pol δ and ε in their capacities to form a holoenzyme structure with the same auxiliary proteins and the fact that both pols are essential gene products for the yeast organism [12,18,19] suggest that pol δ and pol ε might function in chromosomal DNA replication.

In this communication we present the isolation and characterization of a factor from calf thymus that has the properties of RF-C and characterize the pol δ and ε enzymes that both completely rely on SSB, PCNA and RF-C to become functionally active and isolatable holoenzyme forms on singly-DNA primed M13 DNA.

EXPERIMENTAL PROCEDURES

Materials

Nucleotides, column supports and other chemicals. Nucleoside 5′-triphosphates and deoxyribonucleoside 5′-triphosphates, Heparin-Sepharose and FPLC Mono Q were purchased from Pharmacia. Affi-Gel Blue, Bio-Gel HT hydroxylapatite (HAP) and Bio-Gel A-5m were from Bio-Rad. Single-strand DNA (ssDNA) cellulose and the protease inhibitors aprotinin,

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chymostatin, phenylmethanesulfonyl fluoride (PMSF) and N'-p-tosyl-l-lysine chloromethyl ketone (TLCK) were from Sigma. Pepstatin was from Fluka and leupeptin from Boehringer. Amersham was the supplier for all radioactively labelled nucleotides. All other reagents were of analytical grade and purchased from Merck or Fluka.

Nucleic acids. Pharmacia was the supplier of poly(dA) and oligo(dT_{12-18}). *XbaI-EcoRI* polylinker oligo 9013690 (30-mer) was from Biofinex. Single-stranded M13(mp11) DNA was prepared according to [20].

Enzymes and proteins. Calf thymus DNA polymerase δ and ε were isolated as described [21−23] and no crosscontamination could be detected in the final enzyme preparations. Calf thymus PCNA was purified according to [7]. *E.coli* SSB was purified from the overproducer RLM 727 (gift of Martine Defais, CNRS, Toulouse) according to [24].

Buffers. All stock solutions were filtered through nitrocellulose before use (0.45 mm, Schleicher and Schüll). The following buffers were applied: buffer A (20 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM dithiothreitol (DTT), 2 mM EDTA, 0.25 M sucrose, 1 mM ATP, 10 mM benzamidin, 0.5 mM spermidine, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM PMSF, 10 mM sodium bisulfite,1 μg/ml aprotinin, 1 μg/ml chymostatin, 50 μg/ml TLCK); buffer B (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol, 2 mM benzamidin,1 μg/ml pepstatin, 1 mM PMSF, 10 mM sodium bisulfite); buffer C (buffer B, containing 0.01% (v/v) Nonidet P-40); buffer D (50 mM potassium phosphate, pH 7.0, 1 mM DTT, 0.01% Nonidet P-40, 10% (v/v) glycerol, 2 mM benzamidin,1 μg/ml pepstatin, 1 mM PMSF); buffer E (as B, but buffered with 20 mM Tris-HCl, pH 8.0); buffer F (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, 0.01% (v/v) Nonidet P-40, 1 μg/ml pepstatin, 10 mM sodium bisulfite); buffer G (30 mM Tris-HCl, pH 7.5, 20 mM NaCl, 5% (v/v) glycerol, 2 mM DTT, 8 mM $MgCl_2$ and 0.1 mg/ml bovine serum albumin (BSA)).

Methods

Preparation of singly-DNA primed M13 DNA. Single-stranded M13(mp11) DNA (0.1 mg/ml) and 30-mer oligonucleotide (2 μg/ml) were mixed in a buffer containing 10 mM Tris-HCl, pH 7.8, 2.5 mM $MgCl_2$, 0.125 M NaCl, heated at 70°C for 15 min with subsequent slow cooling overnight to room temperature.

DNA polymerase assays. Pol δ and ε were tested, respectively, in an assay containing poly(dA)/oligo(dT_{12-18}) (10:1 base ratio) and Bistris, pH 6.5 as buffer [23] in the presence or in the absence of PCNA (100 ng per assay). 1 unit of enzyme activity corresponds to the incorporation of 1 nmol of dTMP into acid-precipitable material in 60 min at 37°C.

Assay for RF-C. A final volume of 25 μl contained the following components: 40 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.2 mg/ml BSA, 10 mM $MgCl_2$, dATP, dGTP, dCTP each at 50 μM, 15 μM of [^{3}H]dTTP (1500 cpm/pmol), 100 ng of singly-DNA primed M13 DNA, 100 ng of PCNA, 350 ng of *E.coli* SSB, 0.25 U of pol δ and factor to be tested. 1 unit of RF-C activity corresponds to the incorporation of 1 nmol of total dNMP into acid-precipitable material in 30 min at 37°C in the presence of pol δ, SSB and PCNA.

Assay for ATPase. A final volume of 10 μl contained the following components: 40 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.2 mg/ml BSA, 10 mM $MgCl_2$, 50 μM [^{3}H]ATP (600 cpm/pmol), 150 ng of singly-DNA primed M13 DNA, and enzyme as indicated. After incubation for 1 h at 37°C samples were cooled in ice and mixed with 1 μl of 25 mM each of ATP and ADP as markers for UV detection. 10 μl aliquots were spotted on PEI-cellulose thin layer plates (Merck), which were developed in 1 M formic acid/0.5 M LiCl. Regions containing ATP and ADP were cut out and counted.

Purification of RF-C. All isolation steps were performed at 4°C. (*a*) *Isolation of nuclei:* 3 l of buffer A were added to 1 kg of calf thymus, the tissue thawed in small portions and homogenized in a Waring Blendor. The homogenate was centrifugated at 4500×*g* for 15 min and the supernatant was used for isolation of RF-A, PCNA, pols α, δ and ε. The pellet was resuspended in 3 l of buffer A, filtered through two layers of cheese cloth and centrifugated at 4500×*g* for 15 min. (*b*) *Nuclear extract:* Nuclei were suspended in 2 l of buffer B containing 0.2 M NaCl by using the Waring Blendor, stirred for 30 min and centrifugated at 12000×*g* for 30 min. The pellet was resuspended in 2 l of buffer C, containing 0.35 M NaCl, stirred for 1 h and centrifugated at 11000×*g* for 1 h. The supernatant was filtered through four layers of cheese cloth (Fraction I). (*c*) *Affi-Gel Blue chromatography*: Fraction I was adsorbed to a 95 ml Affi-Gel Blue column (4×7.5 cm) equilibrated in buffer C, containing 0.35 M NaCl. The column was washed with 500 ml of equilibrating buffer. RF-C activity was eluted with 300 ml of buffer C, containing 1.0 M NaCl. Fractions, containing RF-C activity were pooled to give the fraction II. (*d*) *HAP*: fraction II was diluted with equal volume of buffer C and loaded to 30 ml HAP column (2.8×5 cm) equilibrated in buffer C, containing 0.1 M NaCl. The column was washed with 100 ml of buffer D followed by 300 ml gradient from 0.05 to 0.5 M potassium phosphate in buffer D. RF-C eluted as a single peak at 0.3 M potassium phosphate. Activity containing fractions were pooled (Fraction III) and dialyzed overnight against buffer C, containing 0.05 M NaCl. (*e*) *ssDNA cellulose*: dialyzed fraction III was loaded to the 10 ml ssDNA cellulose column (1.6×5 cm) equilibrated in dialysis buffer. The column was washed with 30 ml of dialysis buffer. RF-C activity was partially eluted with 50 ml of buffer C, containing 0.1 M NaCl. The rest of RF-C activity was eluted with 100 ml gradient from 0.1 to 0.7 M NaCl in buffer C. Fractions from 0.1 M NaCl wash were pooled to yield fraction IV. (*f*) *FPLC Mono Q*: fraction IV was dialyzed for 3 h against buffer C, containing 0.05 M NaCl and loaded to 5 ml FPLC Mono Q column, equilibrated in buffer E, containing 0.05 M NaCl. The column was washed with 5 ml of equilibrating buffer. RF-C activity was eluted with 30 ml gradient from 0.05 to 0.4 M NaCl in buffer E. Fractions 31−35 were pooled to yield the fraction V. (*g*) *Glycerol gradient:* 100 μl of fraction V was loaded onto a 3.9 ml, 15 to 30% glycerol gradient in buffer F and subjected to centrifugation at 50000 rpm in the TST 60.4 rotor at 4°C for 15 h. The samples were fractionated into 100 μl fractions from bottom to top.

Isolation of initiation and pol holoenzyme complexes with gel filtration. A final volume of 100 ml contained: 40 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.2 mg/ml BSA, 10 mM $MgCl_2$, 600 ng singly-DNA primed M13DNA, 2 μg *E.coli* SSB, 15 ng RF-C, 600 ng PCNA, 1 mM ATP (if added) and 1.25 units of pol δ

or 1.75 units of pol ϵ (if added). After incubation for 2 min at 37°C, the mixture was immediately loaded on a 1.8 ml (0.4×14 cm) Bio-Gel A-5m column equilibrated in buffer G. Gel filtration was carried out at 4°C with a flow rate of 2 ml/h. Two-drop fractions corresponding to about 90 μl were collected. Under these conditions DNA mainly eluted in fractions 5 and 6 and free nucleotides in fractions 12−16. The fractions were assayed for DNA synthesis activity by incubating 22 μl with 3 μl of a solution which yielded finally 50 μM each of dATP, dCTP, dGTP and 15 μM [^{3}H]dTTP (1500 cpm/pmol) and, when indicated, 1 mM ATP, 100 ng PCNA, 0.25 units pol δ or 0.35 units pol ϵ. Incubation was for 30 min at 37°C followed by the determination of acid-precipitable material.

Other methods. DNA products of pol δ and pol ϵ were analysed in 1% alkaline agarose gel as described in [11]. Protein determination and SDS-PAGE were performed according to [25] and [26], respectively. Silver staining was carried out as described in [27].

RESULTS

Purification of RF-C

Replication of singly-DNA primed M13 DNA by calf thymus pol δ was very inefficient and could not be significantly stimulated by PCNA [23], by SSB's (calf thymus RP-A or *E.coli* SSB) or by PCNA and RP-A together (Senn, B. and Hübscher, U., unpublished observations). This was taken to establish an assay that measures DNA synthesis in dependence of pol δ, *E.coli* SSB, PCNA and ATP and a missing activity that we expected to be RF-C according to the data found in the SV 40 *in vitro* replication system [11] or in yeast [28]. Isolated calf thymus nuclei were used as starting material for RF-C purification [11]. High salt extraction from nuclei was found to be essential to obtain RF-C. No RF-C activity was found when 0.2 M NaCl nuclear extract was processed through several chromatographic procedures made as described below. RF-C was extracted from nuclei with 0.35 M NaCl and subsequently purified on Affi-Gel Blue column, HAP, ssDNA cellulose, FPLC Mono Q (Figure 1) and glycerol gradient sedimentation (Figure 2). RF-C eluted from ssDNA cellulose in two forms. Form I eluted at 100 mM NaCl and form II as a broad peak at 300−500 mM NaCl. All subsequent experiments were made with form I. Form II, however, had the same chromatographic behaviour on FPLC Mono Q and in glycerol gradient. The final RF-C preparation contained peptides with similar M_r's to those reported by others [11,28−30]. Seven polypeptides with M_r's of 155, 70, 60, 58, 39 (doublet), 38 (doublet) and 36 kDa cosedimented on a glycerol gradient

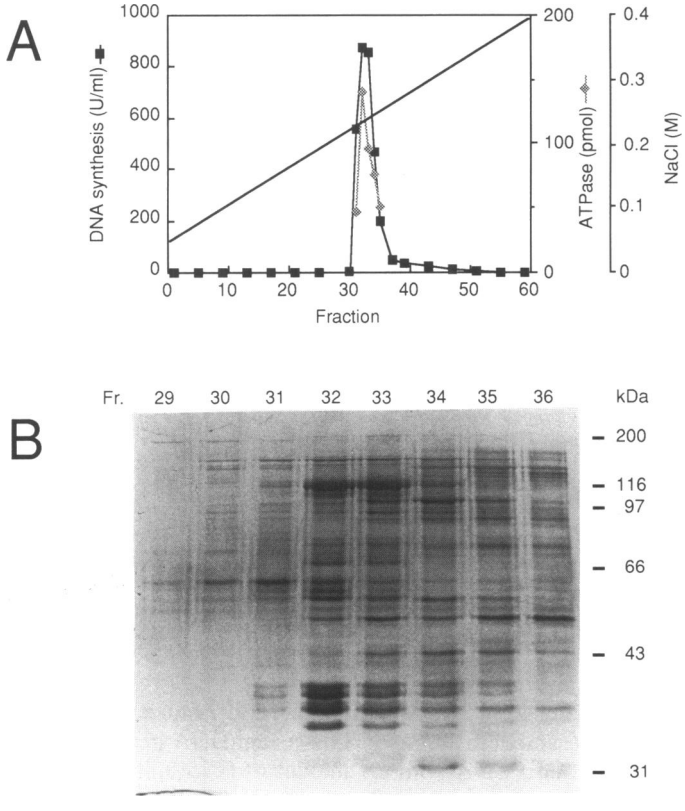

Figure 1. Chromatography of calf thymus RF-C on FPLC Mono Q. Chromatography on FPLC mono Q was carried out as described in Experimental Procedures. RF-C was monitored by complementing pol δ, PCNA and *E.coli* SSB on singly-DNA primed M13 DNA and by determining DNA dependent ATPase. A: Chromatogram: (■) complementation assay, (◆) DNA dependent ATPase. B: SDS-PAGE of active fractions, 10μl were separated on a 8.5% SDS-PAGE as described in Experimental Procedures.

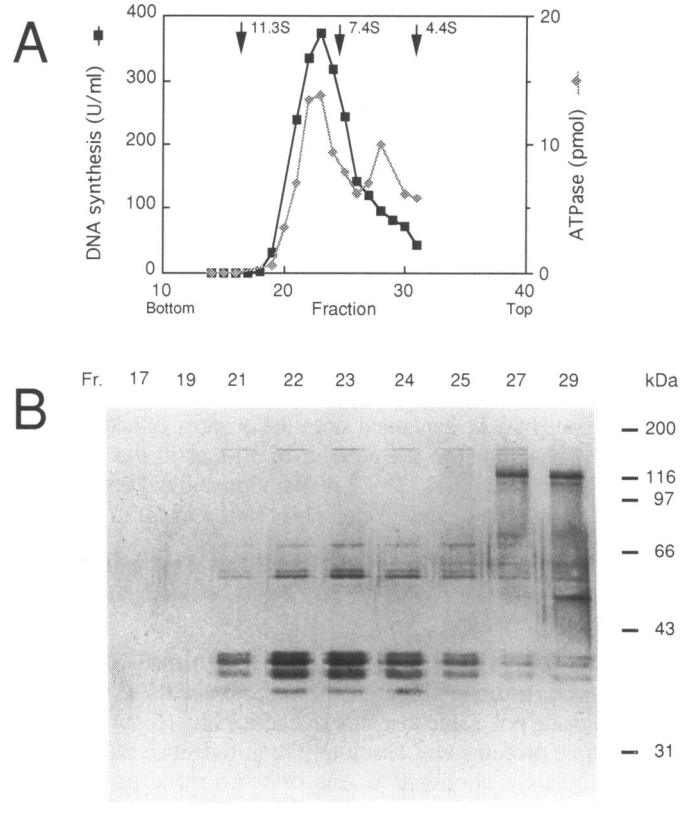

Figure 2. Glycerol gradient centrifugation of calf thymus RF-C. 100 μl (170 units) of RF-C (Fraction V) were loaded on 3.9 ml of a preformed 15−30% glycerol gradient in a buffer containing 25 mM Tris-HCl (pH 7.5), 1 mM DTT, 0,01%(v/v) Nonidet P-40, and 150 mM NaCl. Fractions of 100 μl were collected from the bottom of the tube and monitored for complementation of pol δ, PCNA and *E.coli* SSB on singly-DNA primed M13 DNA and for DNA dependent ATPase as described in Experimental Procedures. Markers used were: catalase (11.3 S), aldolase (7.4 S), and BSA (4.4 S). A: RF-C activity profile, (■) complementation assay, (◆) DNA dependent ATPase ; B: SDS PAGE of RF-C active fractions, 10 μl were separated on a 8.5% SDS PAGE as described in Experimental Procedures.

Table 1. Purification of calf thymus RF-C*.

Fraction	Protein (mg)	Activity (units)	Specific activity (units/mg)
I. Nuclear extract	2900	200,000	69
II. Affi-Gel Blue	150	38,000	250
III. Hydroxylapatite	42	23,000	550
IV. ssDNA cellulose	1.8	6,600	3,700
V. Mono Q	0.19	2,300	12,000
VI. Glycerol gradient	0.026	2,100	81,000

*Purification was started from 1 kg of calf thymus. RF-C activity was monitored by complementation of pol δ, PCNA and *E.coli* SSB on singly-DNA primed M13 DNA.

(Figure 2B). The sedimentation coefficient was 8.2S. Table 1 summarizes the RF-C purification protocol from 1 kg of calf thymus. The overall purification was around 1200-fold with an overall yield of 1% compared to nuclear extract.

Calf thymus RF-C is an ATPase stimulated by DNA and PCNA

ATPase activity followed the RF-C activity in Mono Q chromatography (Figure 1) and glycerol gradient sedimentation (Figure 2). Small but detectable ATPase activity could be measured in the absense of DNA and PCNA. However, ATPase was clearly stimulated up to 7-fold with singly-DNA primed M13 DNA (Figure 3). PCNA in the absence of DNA or in the presence of saturating amount of DNA (150 ng) did not affect the ATPase activity of RF-C. In the presence of an undersaturating amount of DNA (5 ng) PCNA stimulated ATP hydrolysis by RF-C about 2-fold. These data suggested that the mechanism of PCNA stimulation is to increase the affinity of DNA-cofactor to the RF-C complex. Various DNA's were compared for stimulation of ATPase activity (Table 2). The maximal stimulation was observed with ssDNA, no essential difference was observed between unprimed and single-primed M13 DNA. In all cases addition of PCNA had no significant effect. The 2-fold stimulation by PCNA in the presence of 30-mer primer alone may be explained since the quantity of added primer (3 ng) corresponded to the amount of 30-mer in the 150 ng of singly-DNA primed M13 DNA preparation and did not saturated ATPase. These data confirm the observation made in Figure 3.

PCNA, RF-C and ATP are required for DNA polymerases δ and ε for efficient elongation of SSB-coated singly-DNA primed M13 DNA

Pol δ was unable to replicate singly-DNA primed M13 DNA. Simultaneous addition of PCNA, *E.coli* SSB and RF-C to reaction mixture made pol δ able to synthesize efficiently (Figure 4). Each of the three proteins was absolutely required since the synthesis was completely abolished if one of these three proteins was omitted. The dependency on ATP was not absolute, likely due to the fact the RF-C ATPase can also use dATP instead of ATP (data not shown). It has been reported that yeast pol ε [16], calf thymus pol ε [23] and human pol ε [17] are able to replicate to some extent primed M13 DNA alone. However, the combination of the three auxiliary proteins stimulated 10-fold calf thymus pol ε in the presence of ATP and again the dependency on these three proteins was more than 90% (Figure 4). We have found optimal conditions for replication of SSB-coated singly-DNA primed M13 DNA by both pols in the presence of RF-C and PCNA. In both cases 2.5 ng RF-C was sufficient to 'quasy-saturate' 100 ng of

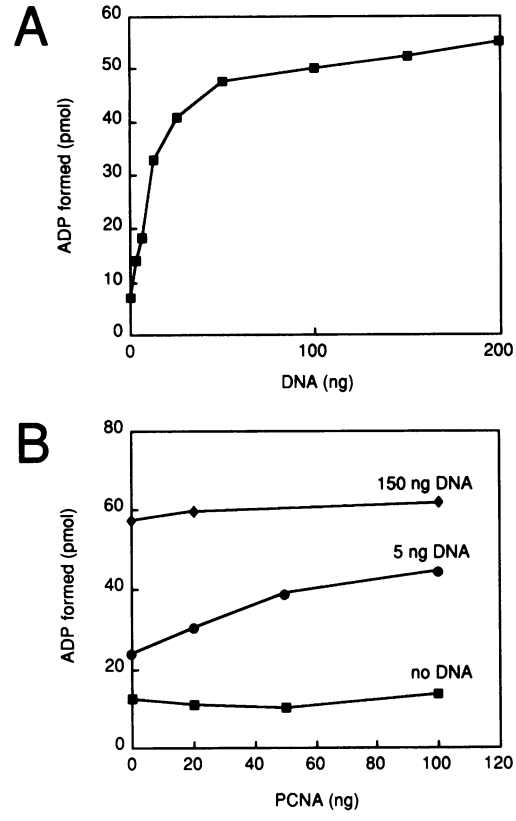

Figure 3. Characterization of calf thymus RF-C ATPase. A: Increasing amounts of singly-DNA primed M13 DNA were tested in an ATPase assay containing 0.7 units RF-C (Fraction V) as described in Experimental Procedures. B: Increasing amounts of PCNA were titrated in an ATPase assay containing 0.7 units RF-C (Fraction V) as described in Experimental Procedures with 0, 5 or 150 ng of singly-DNA primed M13 DNA.

Table 2. Effect of different DNAs on calf thymus RF-C ATPase*.

DNA	ATPase (pmol/h) −PCNA	+ PCNA
None	9	8
Singly-DNA primed M13 DNA	32	34
Unprimed M13 DNA	31	38
30-mer primer	11	21
Poly(dA)/oligo(dT) (10:1 base ratio)	13	19
Activated DNA	16	16

* ATPase was measured in a final volume of 10 μl as described in Experimental Procedures. The assays contained 150 ng of DNA (or 3 ng of 30-mer oligonucleotide corresponding to its content in singly-DNA primed M13 DNA), 0.7 U of RF-C (Fraction V) and, if included, 150 ng of PCNA.

the template. Less PCNA was required for saturation of pol ε. Both pols saturated 100 ng of SSB-coated DNA in the presence of RF-C and PCNA with an amount of 0.35 units.

The experiments so far indicated that both pol δ and ε require the auxiliary proteins PCNA, SSB and RF-C for efficient acting on long single-stranded DNA. The complete systems are therefore called pol δ or pol ε holoenzyme. Product analysis confirmed the data obtained by trichloroacetic acid precipitation (Figure 5). All three proteins were absolutely required for efficient DNA synthesis. Pol δ could not act at all alone and the residual activity of pol ε indicated that this enzyme synthesized rather short

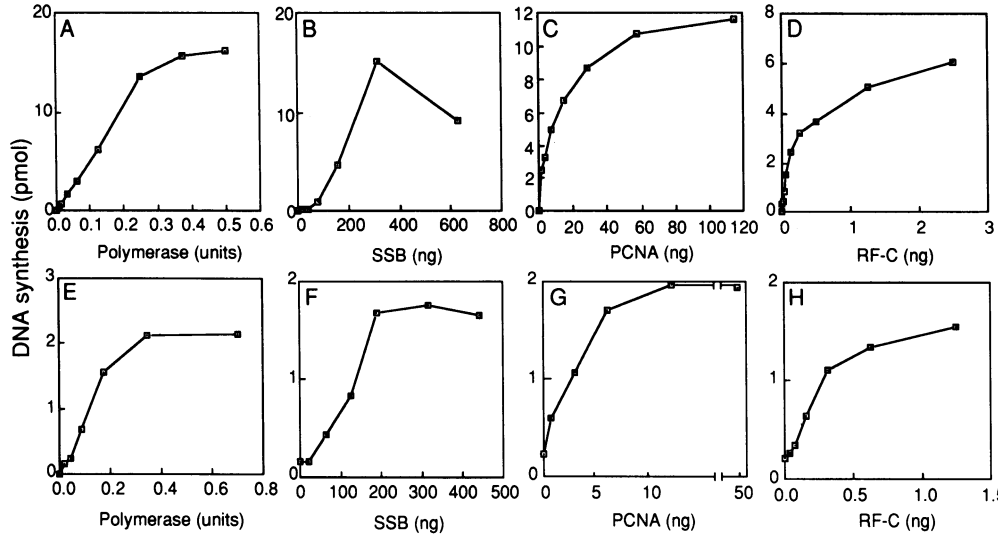

Figure 4. Titration of DNA polymerase δ, ϵ, SSB, PCNA and RF-C on singly-DNA primed M13 DNA. Replication of singly-DNA primed M13 DNA was carried out as described in Experimental Procedures by using the following amounts of proteins and ATP when not varied: pol δ (0.25 units), pol ϵ (0.35 units), SSB (350 ng), RF-C (2.5 ng), PCNA (100 ng for pol δ and 15 ng for pol ϵ) and ATP (1 mM). Figures A–D correspond to pol δ, figures E–H correspond to pol ϵ.

products in the absence of the auxiliary proteins. The extent of DNA synthesis by pol ϵ holoenzyme was lower than for pol δ counterpart (Figures 4 and 5). The extent of pol ϵ stimulation by the auxiliary proteins and the rate of DNA synthesis by pol ϵ holoenzyme was found to vary for primers annealed to different sites of M13 DNA (data not shown). But again total dNMP incorporation in DNA was higher in the case of pol δ holoenzyme.

Holoenzyme complex formation by calf thymus DNA polymerase δ and ϵ with PCNA and RF-C on SSB-coated primed DNA

It has been shown that RF-C in the presence of PCNA and ATP can bind to the 3′-OH primer termini of SSB-coated DNA [31]. In addition a stable DNA-protein complex could be isolated in yeast [16] and human [15]. These data were obtained upon immediate isolation of the complexes by gel filtration. Preincubation of SSB-coated DNA with RF-C, PCNA and ATP yielded a stable complex, that provides DNA synthesis upon addition of only pol δ (Figure 6). When ATP was omitted from the preincubation only simultaneous addition of PCNA, ATP and pol δ restored DNA synthesis. In the experiments omitting ATP the restoration of DNA synthesis was not complete assuming that ATP was not obligoratorily required, but essentially stimulated binding of RF-C to the primer terminus. PCNA could not bind to the DNA/RF-C complex in the absence of ATP. When SSB-coated DNA was preincubated with RF-C, PCNA, pol δ (or pol ϵ) and ATP, isolatable complexes could replicate DNA upon addition of dNTP's only (Fig. 7). However, a further increase of DNA synthesis was observed when pol δ or pol ϵ were added to the filtrated complexes. This meant that both pol δ and pol ϵ were able to form stable holoenzyme complexes, but the binding of pol δ and ϵ might partially be reversible. When DNA was preincubated with RF-C, PCNA and pol δ (or pol ϵ) in the absence of ATP only simultaneous addition of pol, PCNA and ATP restored the DNA synthesis. These data indicated that pol δ as well as pol ϵ could be retained by SSB-coated DNA only via the specific holoenzyme complex.

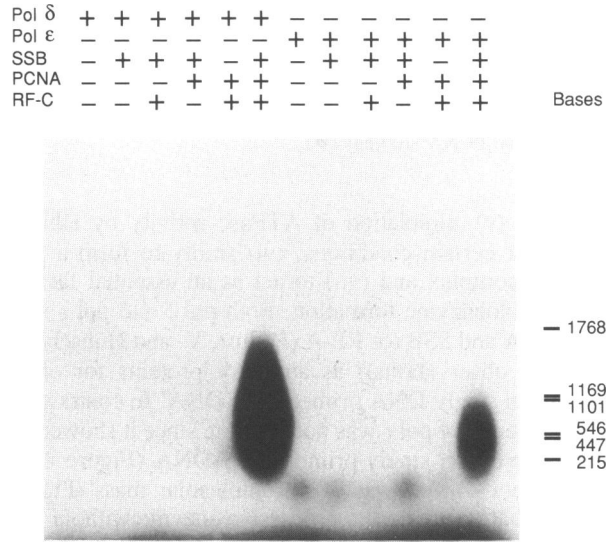

Figure 5. Replication of singly-DNA primed M13DNA is dependent on SSB, PCNA, RF-C. Replication of singly-DNA primed M13 DNA was carried out as described in Experimental Procedures by using the following amounts of proteins and ATP: pol δ (0.25 units), pol ϵ (0.35 unit), *E.coli.* SSB (350 ng), RF-C (2.5 ng) and PCNA (100 ng). For product analysis the reaction was stopped, prepared for electrophoresis and separated on a 1% alcaline agarose gel as outlined in the Experimental Procedures.

DISCUSSION

Calf thymus RF-C has been purified 1200 fold with a complementation assay that allowed DNA synthesis on singly-DNA primed M13 DNA in the presence of pol δ, PCNA, *E.coli* SSB and ATP. The purified protein had *bona fide* properties typical for RF-C such as (i) extractability from nuclei, (ii) chromatographic behaviour, (iii) subunit composition, (iv) copurification with ATPase activity in the late stages of

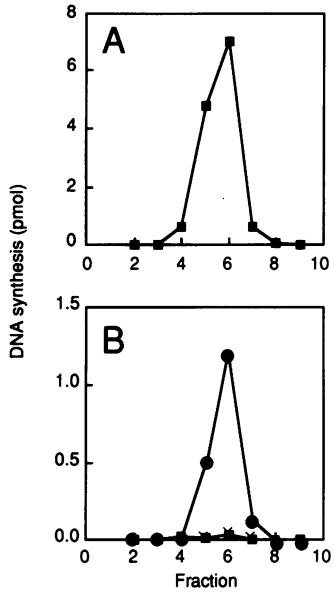

Figure 6. Gel filtration of primer recognition complex formed by calf thymus RF-C and PCNA. Primer recognition complex formation on singly-DNA primed M13 DNA was carried out, the products fractionated on a Bio-Gel A-5m column and the fractions from the column supplemented with a dNTP mixture as described in Experimental Procedures. A: Complex formation was performed in presence of 1 mM ATP; the fractions from the column were supplement with pol δ (0.25 units). B: Complex formation was measured in the absence of ATP. The fractions from the column were supplemented first with pol δ (0.25 units) (■), second with pol δ (0.25 units) and ATP (1 mM) (×) and third with pol δ (0.25 units), ATP (1 mM) and PCNA (100 ng) (●).

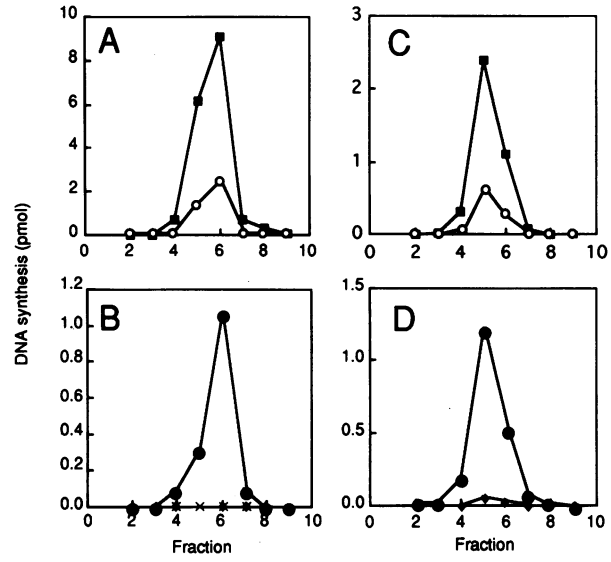

Figure 7. Gel filtration of calf thymus DNA polymerases δ and ε holoenzyme complexes. Holoenzyme complex formation on singly-DNA primed M13 DNA was carried out, the products fractionated on a Bio-Gel A-5m column and the fractions supplemented with a dNTP mixture as described in Experimental Procedures. A: Holoenzyme complex formation was perfomed in the presence of ATP and the fractions from the column supplemented with dNTP's only (○) or with 0.2 units of pol δ (■). B: Holoenzyme complex formation was perfomed in the absence of ATP and the fractions from the column were supplemented with dNTP's plus first,with pol δ (0.25 units) and ATP (1 mM) (×), second, with PCNA (100 ng) and ATP (1 mM) (♦) and third, with pol δ (0.25 units), PCNA (100 ng) and ATP (1 mM) (●). C: As A, but pol δ was replaced by 0.35 units of pol ε. D: As B, but pol δ was replaced by 0.35 units of pol ε.

purification, (v) stimulation of ATPase activity by DNA and PCNA under certain conditions, (vi) ability to form a primer recognition complex and (vii) to act as an essential factor for successful holoenzyme formation. Both pol δ and pol ε require RF-C, PCNA and SSB (or RP-A (Podust, V. and Hübscher, U., unpublished observations)) as auxiliary proteins for efficient replication on singly-DNA primed M13 DNA. In contrast to pol δ the dependency of pol ε was not absolute since it showed some residual activity on singly-primed M13 DNA (Figure 4). The product of pol ε alone was of low molecular mass (Figure 5) suggesting that pol ε is not a processive enzyme without RF-C, PCNA and SSB. Under optimal replication conditions the rate of DNA synthesis of pol ε holoenzyme appeared to be 10-fold lower than for pol δ holoenzyme counterpart (see also below). On SSB-coated singly-DNA primed M13 DNA RF-C could bind to the primer terminus and together with PCNA and ATP form a primer recognition complex (Figure 6). Upon addition of pol δ or pol ε to RF-C, PCNA and ATP stable isolatable holoezyme complexes were formed (Figure 7). Upon addition of pol δ or pol ε to the isolated holoenzyme/DNA complexes a 3-fold increase of DNA synthesis could be observed indicating that the holoenzyme complex formation is a dynamic process.

What might the lower rate of pol ε holoenzyme mean in regard to its *in vivo* function? Nethanel and Kaufmann [32] proposed that two pol's are required for the replication of the lagging strand. In a region which they called 'Okazaki zone' several Okazaki fragments could be initiated simultaneously by several pol α/primase complexes. Pol α would synthezise very short Okazaki fragment and immediately would allow the RNAse H to remove the RNA-primer. The remaining DNA part is used for further elongation. Pol ε holoenzyme would be a candidate for the completion of the Okazaki fragments within a zone. For this the enzyme rather would need an accurate gap completion than a high replication speed. Pol δ on the other hand, presumably acting on the leading strand has to be very processive and fast. Thus we would favour the model introduced by Burgers [16] that proposes pol δ as the leading and pol α/primase and pol ε as the two lagging strand replicases. An alternative explanation for the lower rate might be that we still miss additional factors. Indeed we found that replication products of pol ε holoenzyme were different when cruder pol ε and RF-C fraction were used (Podust, V. and Hübscher, U., unpublished observations).

In conclusion the existence of active and isolatable pol δ and ε holoenzyme forms from the calf thymus tissue is of advantage since this gland is a rich and cheap source to obtain large quantities of all these replication factors described. Biochemical analysis to test the model that two pol's act at the lagging strand [32] are now possible for bovine enzymes and auxiliary proteins. Finally, functional experiments at model replication forks with pol δ and pol ε holoenzymes and pol α/primase in the presence of RP-A, alpha accessory factor [33] and one or more cellular DNA helicases that have very recently been identified from the same tissue [34] might tell us how three replicative DNA polymerases might share their roles at a mammalian replication fork.

ACKNOWLEDGEMENTS

This work was supported by the Swiss National Science Foundation (grants 31.28592.90 and 31.30298.90), by the Swiss Cancer Society, by the Ciba-Geigy Jubiläumsstiftung, by the

Bonizzi Theler Stiftung and by the Kanton of Zürich. The authors thank Barbara Senn for her initial work in this project and Peter Burgers for important suggestions.

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