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A complex between replication factor A (SSB) and DNA helicase stimulates DNA synthesis of DNA polymerase α on double-stranded DNA

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A helicase-like DNA unwinding activity was found in highly purified fractions of the calf thymus single-stranded DNA binding protein (ctSSB), also known as replication protein A (RP-A) or replication factor A (RF-A). This activity depended on the hydrolysis of ATP or dATP, and used CTP with a lower efficiency. ctSSB promoted the homologous DNA polymerase α to perform DNA synthesis on double-stranded templates containing replication fork-like structures. The rate and amount of DNA synthesis was found to be dependent on the concentration of ctSSB. At a 10-fold mass excess of ctSSB over double-stranded DNA, products of 200–600 nucleotides in length were obtained. This comprises or even exceeds the length of a eukaryotic Okazaki fragment. The ctSSB-associated DNA helicase activity is most likely a distinct protein rather than an inherent property of SSB, as inferred from titration experiments between SSB and DNA. The association of a helicase with SSB and the stimulatory action of this complex to the DNA polymerase α-catalyzed synthesis of double-stranded DNA suggests a cooperative function of the three enzymatic activities in the process of eukaryotic DNA replication.

Calf thymus; DNA replication; DNA unwinding; SSB protein; Single-stranded DNA-binding protein

1. INTRODUCTION

During DNA replication, opening of the double-stranded DNA helix is a key step to providing a single-stranded template from which the replicative DNA polymerases can copy [1]. This role is fulfilled by the cooperative action of two proteins, DNA helicase and single-stranded DNA binding protein (SSB). DNA helicase disrupts, in an energy-consuming manner, the hydrogen bonds of the two complementary DNA strands. SSB protein stabilizes the newly generated single-stranded DNA and prevents reannealing to the energetically favored duplex structure. Eukaryotic SSBs consist of three polypeptides with molecular weights of about 70, 32, and 14 kDa [2–4]. The corresponding genes are all essential for cellular viability [5]. Until no,w as many as eight different DNA helicases have been identified in higher eukaryotes (see e.g. [6] and references therein). It is not yet clear which of these might be involved in the process of eukaryotic DNA replication. The finding of a functional interaction between SSB and helicase on the one hand and a replicative polymerase on the other hand would certainly aid the identification of a replicative DNA helicase.

In this report, we demonstrate that highly purified fractions of ctSSB containe DNA helicase activity. Furthermore, the helicase-containing SSB allows the homologous DNA polymerase α to replicate dsDNA.

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Abbreviations: SSB, single-stranded DNA-binding protein; ctSSB, SSB from calf thymus; dsDNA, double-stranded DNA; PAGE, polyacrylamide gel electrophoresis; pol-α, DNA polymerase α; SDS, sodium dodecylsulfate; RF-A, replication factor A, RP-A, replication protein A.

2. MATERIALS AND METHODS

2.1. *Materials*

ctSSB was purified to > 95% homogeneity by chromatography on DEAE-cellulose, blue-Sepharose, ssDNA cellulose and FPLC Mono Q [7]. Calf thymus pol-α was immunoaffinity purified as described [8]. Klenow polymerase was from New England Biolabs. T4 DNA polymerase was a kind gift of L. Reha-Krantz, University of Alberta, Canada. Calf thymus terminal transferase (TdT) was from Stratagene. Nucleotides, nucleic acids and chemicals were as described [9].

2.2. *Construction of a dsDNA template with replication fork-like termini*

pUC19 plasmid DNA (40 μg) was linearized with *Sca* I (40 U). The resulting blunt ends were converted into defined 5′ overhangs by T4 DNA polymerase in the presence of dCTP as the only nucleoside triphosphate [10]. The recessed 3′-OH ends were elongated by terminal transferase and dATP to yield oligo(dA) tails with a mean length of around 50 [11]. The phenol-extracted DNA was annealed to oligo$(dT)_{12-18}$ and used as the substrate for dsDNA replication.

2.3. *Assay for double-strand DNA replication*

The assay mixture (20 μl) contained 20 mM Tris/acetate, pH 7.3, 5 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mM of each of the dNTPs, 50–100 cpm/pmol [α-^{32}P]dATP, 0.8 μg of the tailed dsDNA

and 1 U of pol-α. Incubation was at 37°C for 60 min. dNTP incorporation was measured by acid precipitation as described [12]. The lengths of synthesized DNA products were analyzed on a 4% polyacrylamide gel containing 9 M urea.

2.4. *Other methods*

DNA helicase and DNA-dependent ATPase assays were performed as described [9].

3. RESULTS AND DISCUSSION

To study the strand displacement ability of DNA polymerase α under various conditions we have designed a linear dsDNA that contains replication fork-like termini (Fig. 1). As expected, this DNA was efficiently used by the strand-displacing Klenow polymerase, leading to replication products some 600–1,000 nucleotides in length (Fig. 2, lane 1). By contrast, pol-α synthesized 20–80 nucleotides (Fig. 2, lane 2), which is the average length distribution of oligo(dA) tails at the fork-like termini. Thus, pol-α alone copied the oligo(dT)-primed oligo(dA) tails but stalled DNA synthesis at duplex structures. However, when ctSSB was added, pol-α replicated former double-stranded regions of the template (Fig. 2, lanes 3–7), resulting in a 7.5-fold stimulation of the reaction rate at a 10-fold mass excess of SSB over DNA (Fig. 2, lane 6). Products with a length distribution of 90–200 were formed when SSB exceeded the mass of DNA 2.5–5-fold (Fig. 2, lanes 3–4). At a 10–12.5-fold mass excess of SSB over DNA (Fig. 2, lanes 6–7) 200–600 nucleotide long products were formed. The observed product lengths are in agreement with the proposed role of pol-α as the lagging strand DNA polymerase, since eukaryotic Okazaki fragments are about 200 nucleotides long [13]. Stimulation of pol-α by SSB and a comparable distribution of product lengths has also been found upon in vitro replication of SV40 origin-containing plasmids [14]. The latter reaction, however, required the helicase function of SV40 large T-antigen.

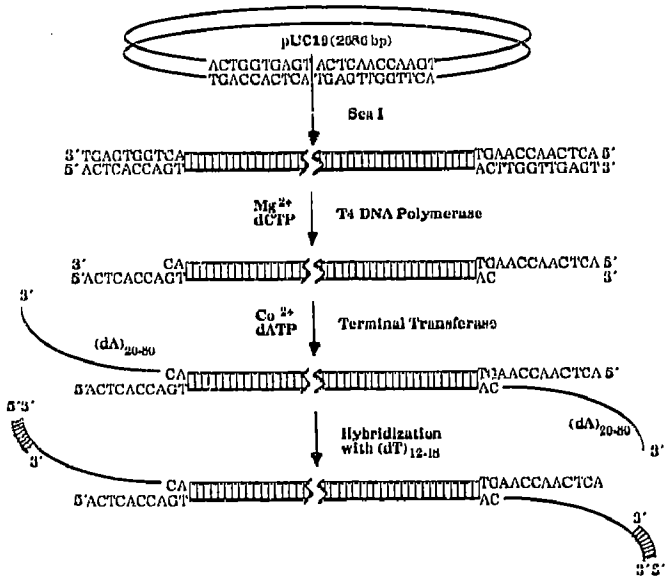
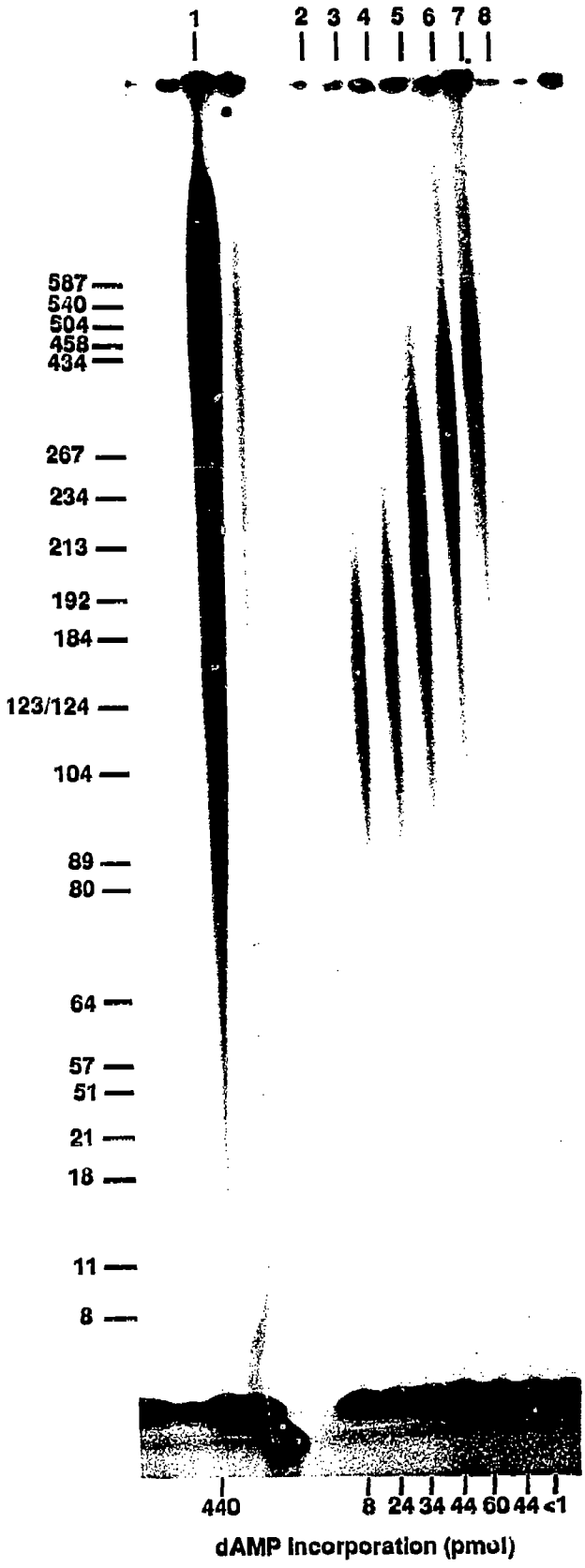


Fig. 1. Construction of a double-stranded DNA template terminated with a replication-fork like structure. For details see Materials and Methods.

Hence, we suspected that a DNA helicase activity might have been part of our SSB fraction. To test for this possibility we employed a strand-displacement assay that had recently been used for the detection of helicases [9]. This assay revealed that ctSSB contained DNA unwinding activity (Fig. 3, upper panel). Moreover, oligomer unwinding required the presence of ATP or dATP (Fig. 3, lower panel). The amount of DNA unwinding was clearly dependent on the ratio of ctSSB to ssDNA. Significant ATP-dependent unwinding was observed when ctSSB covered 5–10% of the ssDNA. At full saturation, ATP-dependent DNA unwinding was optimal (Fig. 3, lower panel). Higher concentrations of ctSSB led to an inhibition of the unwinding reaction (Fig. 3). Inhibition of DNA unwinding at high concentrations of SSB is not in agreement with the assumption that the helicase is an intrinsic part of SSB. Rather, this type of inhibition can be explained by assuming that a co-purifying but distinct helicase competes with SSB for binding sites at partial duplexes. A comparable phenomenon has recently been observed: a DNA helicase from HeLa cells was strongly stimulated by human SSB up to a concentration where SSB completely covered the ssDNA template. At higher concentrations SSB became inhibitory for the unwinding reaction of the distinct helicase [15].

The nucleotide requirements of the DNA unwinding activity associated with ctSSB were further analyzed. The helicase preferred ATP and dATP as nucleotide cofactors; CTP and possibly also UTP could be used but resulted in less efficient unwinding (Fig. 4). The other nucleoside triphosphates did not sustain DNA unwinding. The observed nucleotide specificity is very similar to the nucleotide preferences of the SSB-dependent helicase from HeLa cells [15]. Because of its functional interaction with SSB and apparent species specificity, this helicase is a likely candidate for co-purification with its homologous SSB. In such a case, the detection of this helicase would be difficult because both the largest subunit of the SSB trimer and the helicase have very similar molecular weights. Furthermore, the sedimentation coefficient of this helicase is 5.2 S [15], essentially the same value found for calf thymus SSB [7]. This coincidence might explain why we could not remove the helicase from ctSSB by glycerol gradient centrifugation (data not shown).

Two reports deal about interactions between SSB and DNA helicases from calf thymus [6,16]. The bovine helicases A and E are both stimulated by ctSSB. Moreover, they both bear many similarities to the SSB-dependent helicase from HeLa cells [6,16,17]. Interestingly, helicase A has been reported to co-purify with



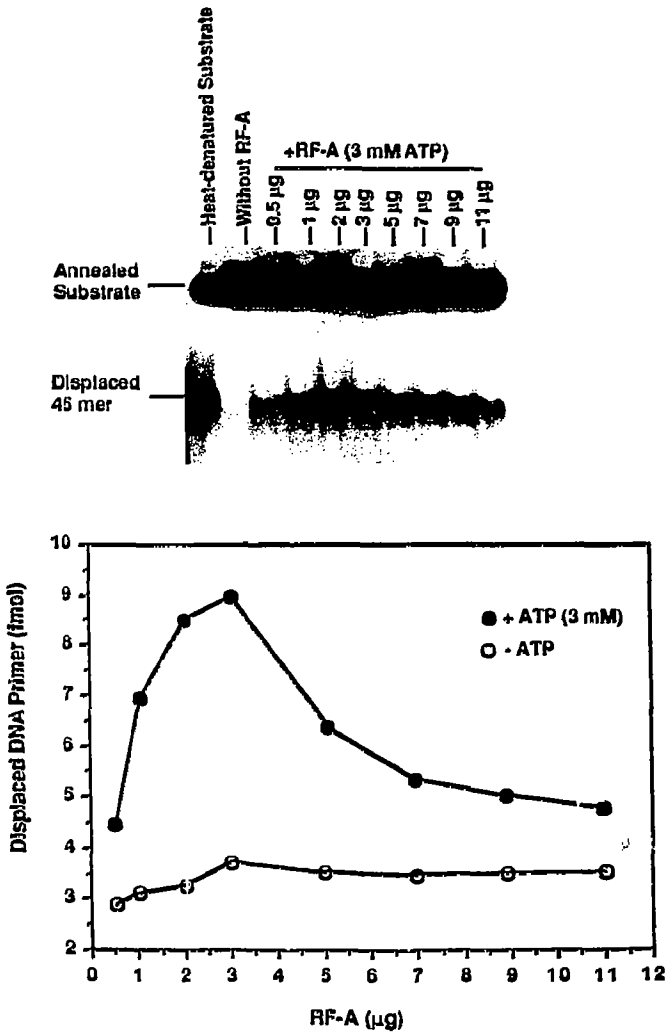


Fig. 3. ATP-dependent DNA unwinding in the presence of increasing amounts of purified calf thymus RF-A protein. DNA helicase activity was measured as described [9] with various amounts of RF-A as indicated. DNA unwinding was quantified by excising the radioactive bands from the gel and measuring their corresponding radioactivity by scintillation counting.

DNA polymerase α [17], while helicase E co-purifies over several steps with DNA polymerase ε* [16]. Co-purification of DNA helicase with RF-A on the one hand and DNA polymerase on the other hand suggests

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Fig. 2. DNA polymerase α-catalyzed DNA synthesis on double-stranded DNA. The reaction was performed as described in Materials and Methods. Lane 1, DNA synthesis performed by 10 U Klenow polymerase (equivalent to 50 pol-α U). Lane 2, 1 U pol-α without SSB. Lanes 3–7, 1 U pol-α and 2 µg (lane 3), 4 µg (lane 4), 6 µg (lane 5), 8 µg (lane 6) and 10 µg (lane 7) of ctSSB. Lane 8, 10 µg of ctSSB without pol-α. *Hae*III-digested pBR322 (Boehringer, Mannheim) was used as a lengths standard. The amount of dAMP incorporation was quantified by taking portions of the reaction mixture and measuring the acid insoluble radioactivity. These values are indicated at the bottom of the autoradiograph.

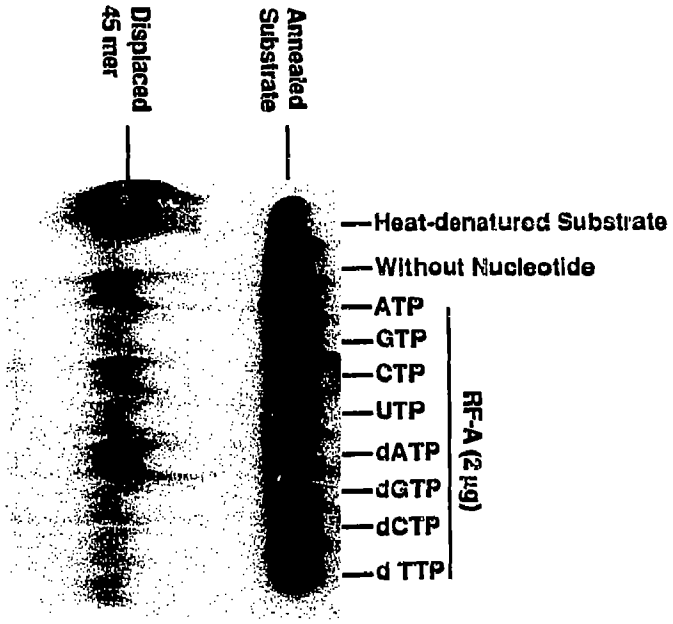

Fig. 4. Nucleotide requirements of the ctSSB-associated DNA helicase. DNA helicase activity was measured in the presence of 3 mM of the indicated nucleoside triphosphates by using 2 μg of ctSSB, basically as described elsewhere [9].

physical interactions between these replicative enzymes. The efficient replication of double-stranded structures by a complex consisting of SSB, a helicase and pol-α, as shown in this study, points to a functional association of the three enzymes and suggests a physiological role of this particular helicase in the process of eukaryotic DNA replication.

After this work had been finished, ctSSB was shown to unwind DNA passively, i.e. in a reaction that did not require an energy-delivering nucleotide cofactor [18]. Thus, passive unwinding might have been responsible for the observed stimulatory effect of ctSSB on pol-α. However, according to Georgaki et al., passive unwinding is extremely sensitive to the presence of Mg^{2+}. In our work, the strand displacement assay was performed at 5 mM Mg^{2+}, a concentration at which passive unwinding of long stretches of DNA was undetectable [18].

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