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Calf thymus DNA helicase F, a replication protein A copurifying enzyme

Anthi Georgaki, Narendra Tuteja[1], Birgit Sturzenegger and Ulrich Hübscher*
Department of Veterinary Biochemistry, University of Zürich – Irchel, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland and [1]International Centre for Genetic Engineering and Biotechnology, Area Science Park, Padriciano 99, I-34012 Trieste, Italy

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ABSTRACT

A DNA helicase from calf thymus, called DNA helicase F, copurified with replication protein A through several steps of purification including DEAE-Sephacel, hydroxyapatite and single stranded DNA cellulose. It is finally separated from replication protein A on FPLC Mono Q where the DNA helicase elutes after replication protein A. Characterization of the DNA helicase F by affinity labeling with [α^{32}P]ATP indicated that the enzyme has a catalytic subunit of 72 kDa. Gel filtration experiments suggested that DNA helicase F can exist both in a monomeric and an oligomeric form. The enzyme unwinds DNA in the 5′→3′ direction in relation to the strand it binds. All eight deoxyribonucleoside- and ribonucleosidetriphosphates could serve as an energy source. Testing a variety of DNA/DNA substrates demonstrated that the DNA helicase F preferentially unwinds very short substrates and is slightly stimulated by a single stranded 3′-tail. However, replication protein A allowed the DNA helicase to unwind much longer DNA substrates of up to 400 bases, indicating that the copurification of replication protein A with the DNA helicase F might be of functional relevance.

INTRODUCTION

DNA helicases are enzymes that can transiently melt double stranded DNA by using the energy of ribonucleoside or deoxyribonucleoside 5′-triphosphate hydrolysis. The variety of different needs for the double stranded DNA to be opened appears the reason why a set of more than ten different DNA helicases have been found initially in single cell organism like *Escherichia coli* (reveiwed in ref. 1) and more recently also in eukaryotic, including human cells (reviewed in ref. 2). These needs include DNA transactions such as DNA replication, DNA repair, DNA recombination and transcription.

Several different strategies led to the finding of a whole set of DNA helicases from different eukaryotes (reviewed in ref. 2). The first approaches involved the isolation of proteins with DNA dependent ATPase activity since DNA helicases use the energy from ATP hydrolysis for the melting of the double helix (3). More progress was achieved by using a strand displacement assay first described for the characterization of helicases of *Escherichia coli* (4). During the purification of DNA polymerases calf thymus extracts were prefractionated thus decreasing the presence of nucleases, and this led to the identification of eight DNA helicases. For example, in a preparation of DNA polymerase δ which was able to perform strand displacement synthesis, a DNA helicase could be isolated, which confers this property to the DNA polymerase (5). Similarly DNA helicases have been found to copurify initially with DNA polymerases α (6) and ε (7), the former called DNA helicase A and the latter DNA helicase E, respectively. With refined isolation schemes for the simultaneous purification of DNA polymerases α, δ, and ε (8) four different DNA helicases A, B, C and D were found and initially characterized from calf thymus (9). By concentrating on enzymes present in the nuclear fraction of a calf thymus extract two additional DNA helicases could be distinguished (10). They seem to be different from the six cytosolic enzymes mentioned. Taken together there are eight different DNA helicases from calf thymus described so far, a number approaching that from *Escherichia coli* (11).

Moreover a similar strategy has been systematically employed for the isolation of several DNA helicases from human cells, which so far has succeded in the characterization of seven human enzymes (12–18). In addition two functional approaches have been chosen aimed at the isolation of enzymes involved in the initiation of replication. When looking at proteins from human cell extracts binding to the dihydrofolic reductase origin of replication a protein of 100 kDa (RIP 100) was found to contain DNA helicase activity (19). This enzyme interacts with another origin binding protein, the RIP60, and is assumed to play a role during the initial opening of the double helix.

In yeast *Saccaromyces cerevisiae* the best characterized DNA helicase is the Rad3 protein (1). Other yeast DNA helicases are the SRS2 enzyme (20) and the transcription factor BTF2 (also called TFIIH, ref. 21). A DNA helicase copurifying with replication factor C has recently been identified (22) as well as an enzyme called DNA helicase III (23).

*To whom correspondence should be addressed

The best characterized DNA helicases are of viral origin. They include (i) the SV40 large T antigen (reviewed in ref. 24), (ii) the Herpes Simplex Virus type 1 UL9 DNA helicase and the heterotrimeric DNA primase-helicase consisting of the polypeptides from UL5, UL8 and UL52 (reviewed in ref. 25) and finally (iii) the bovine papilloma virus E1 DNA helicase (26, 27).

In this communication we characterize a novel DNA helicase, called DNA helicase F, from calf thymus as an enzyme that copurifies with the essential replication and repair protein RP-A. The fact that RP-A stimulated the DNA helicase to unwind long stretches of DNA suggest that the copurification of these two proteins might be of functional relevance.

MATERIALS AND METHODS

Materials

Nucleotides, column supports and other chemicals. Nucleoside 5′-triphosphates and deoxyribonucleoside 5′-triphosphates, were purchased from Pharmacia. Amersham was the supplier for all radioactively labelled nucleotides. All other reagents were of analytical grade and purchased from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland).

Nucleic acids. All oligonucleotides used in this work have either been described earlier (9, 18). Singly primed M13 DNA was prepared according to Podust *et al.* (28).

Enzymes and proteins. DNA polymerase δ and DNA helicase A were isolated from calf thymus as described in ref. 8 and ref. 9, respectively. Calf thymus PCNA and RF-C were purified according to Prelich *et al.* (29) and Podust *et al.* (28), respectively and RP-A according to Georgaki and Hübscher (30). *Escherichia coli* SSB was purified from the overproducer RLM 727 (gift of Martine Defais, CNRS, Toulouse) according to ref. 31.

Methods

Preparation of the DNA helicase and the directionality substrates. The 24-mer and the long >400-mer DNA helicase substrates to determine DNA helicase and DNA unwinding by RP-A were prepared and radioactively labeled as described (9). The directionality substrates was constructed by hybridizing a 30-mer *Xba*I−*Eco*RI polylinker oligonucleotide to ssM13 DNA as outlined in (9). All other DNA helicase substrates tested (see Figure 6A) have been described (18).

Assays for RP-A. The fate of RP-A during isolation was followed with three assays according to ref. 30: (i) by testing individual fractions with a polyclonal antibody raised in chicken; (ii) by *in vitro* complementation of DNA polymerase δ, PCNA and RF-C on a singly-DNA primed M13 DNA template as described below and (iii) with a DNA unwinding assay: Unwinding of the short 24-mer substrate was carried out in a final volume of 25 μl containing: 20 mM Tris−HCl (pH 7.5), 4%(w/v) sucrose, 8 mM DTT, 80 μg/ml bovine serum albumin, 10 ng DNA substrate (3000 cpm/pmol) and protein to be tested. Incubation was for 60 min at 37°C unless otherwise mentioned. The reaction was stopped, the products separated and autoradiographed as described (9). Quantification was performed by using a Phosphor Imager (Molecular Dynamics).

DNA helicase assay. DNA helicase activity assays were performed according to Thömmes and Hübscher (6) in a final volume of 25 μl containing: 20 mM Tris−HCl (pH 7.5), 4% (w/v) sucrose, 8 mM DTT, 80 μg/ml bovine serum albumin, 1 mM $MgCl_2$, 1 mM ATP, 10 ng DNA substrate (3000 cpm/pmol) and DNA helicase to be tested. Incubation was for 60 min at 37°C. The reaction was stopped, the products separated, autoradiographed as described (9). Quantification was performed as above.

Purification of DNA helicase F. The DNA helicase F was isolated from calf thymus according to an optimized isolation protocol for RP-A (30). Briefly, the purification procedure included the five steps DEAE-Sephacel, hydroxyapatite, ssDNA cellulose, FPLC Mono Q (30) and FPLC Superose. The fractions (49−59) of the FPLC Mono Q column containing the DNA helicase activity (Figure 1) were pooled and loaded onto a FPLC Superose 6 column (HR 10/30) equilibrated with a buffer containing 20 mM Tris−HCl (pH 8.0), 10%(v/v) glycerol, 0.02%(v/v) Nonidet P-40, 2 mM EDTA, 1 mM DTT, 10 mM

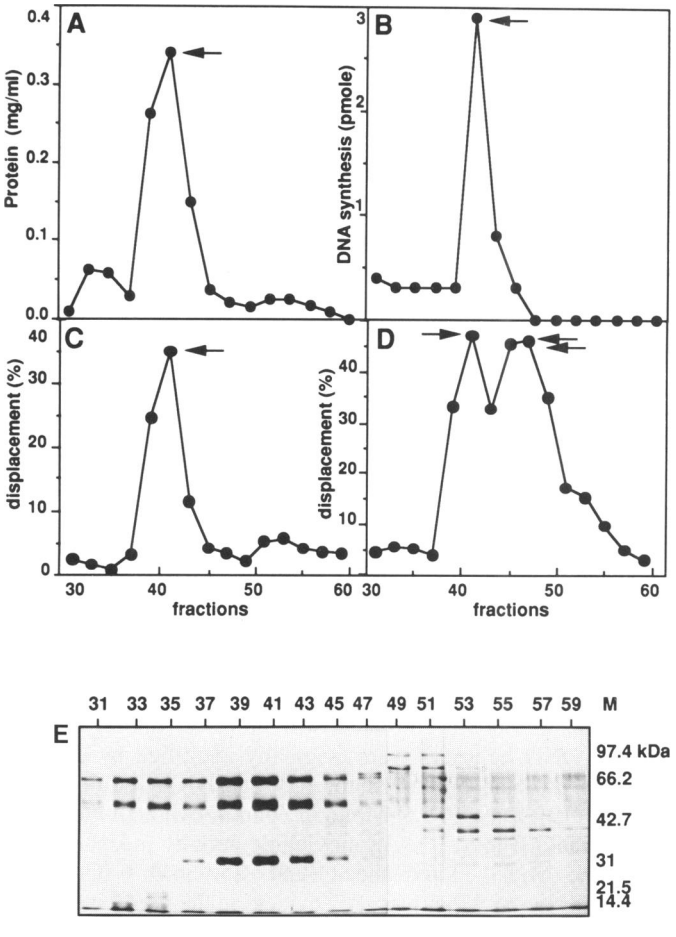

Figure 1. Chromatography on FPLC-Mono Q separates calf thymus DNA helicase F from RP-A. Chromatography on FPLC Mono Q was performed as described in Materials and Methods. Every second fraction was tested for protein concentration (**A**), for stimulation of DNA polymerase δ holoenzyme in the presence of PCNA and RF-C on primed M13 DNA (**B**), for DNA unwinding in the absence of ATP and $MgCl_2$ (**C**) and for DNA helicase activity in the presence of 1mM each of ATP and $MgCl_2$ (**D**). (**E**): 2 μl of each second fraction were loaded onto a 12.5% SDS-PAGE, separated and silver stained.

sodiumbisulfite, 1 mM PMSF and 1 μg/ml each of pepstatin and leupeptin. The DNA helicase activity was eluted in the same buffer at a flow rate of 0.5 ml/min. As marker proteins bovine serum albumin (M_r 67 kDa) and lactate dehydrogenase (M_r 142 kDa) were used. The DNA helicase active fractions were stored as individual fractions in liquid nitrogen until further use.

Photo affinity labeling. Covalent labeling of DNA helicase F was performed essentially as described by Tuteja *et al.* (18). Briefly, 10 μl of protein fraction (700 ng) was used in a 20 μl of reaction volume containing 12 mM Hepes (pH 7.9), 60 mM KCl, 1 mM $MgCl_2$, 6 mM DTT, 0.5 μl of [α^{32}P]ATP (specific activity 400 Ci/mmol) and 3 μg/ml unlabeled helicase substrate (17 bp annealed to ss M13 DNA having 15 nt ss tails at 5′ and 3′ ends, see Figure 6A, panel a). The mixture was irradiated with a 4 Watt, 254 nm UV light at a distance of 5 cm for 30 min in ice followed by another 10 min incubation at room temperature without irradiation. The products were separated by 10% SDS-PAGE and the radiolabeled polypeptide was visualized by autoradiography.

In vitro replication of singly primed M13DNA. This replication assay was carried out as described (28). A final volume of 25 μl contained the following components: 40 mM Tris−HCl (pH 7.5), 1 mM DTT, 0.2 mg/ml bovine serum albumin, 10 mM $MgCl_2$, dATP, dGTP, dCTP each at 50 μM, 15 μM of [^{3}H]dTTP (1500 cpm/pmol), 1mM ATP, 100 ng of singly-DNA primed M13 DNA, 100 ng of PCNA, 10 ng RF-C, 0.25 U of DNA polymerase δ and RP-A to be tested. *Escherichia coli* SSB (600 μg) was used as a positive control. The reactions were incubated for 60 min at 37°C and DNA synthesis measured as described (28).

Other methods. Protein determinations and SDS-PAGE were carried out according to ref. 32 and ref. 33, respectively. Silver staining was performed accoding to the protocol of Arezzo and Rose (34).

RESULTS

A DNA helicase from calf thymus copurifies with RP-A through several purification steps and finally separates on FPLC mono Q from the DNA unwinding activity of RP-A

In the course of isolation of calf thymus RP-A we realized that a DNA helicase has a tendency to copurify with this protein (30). This observation was consistent with an earler finding that calf thymus DNA helicases were originally identified by using an isolation protocol optimized for RP-A (ssDNA cellulose flow through) (9). On a FPLC-Mono Q column two activities could be separated (Figure 1). An unwinding activity independent of ATP and $MgCl_2$ eluted ahead (230−250 mM NaCl) of an unwinding activity that was only evident in the presence of ATP and $MgCl_2$ (280−350 mM NaCl) (compare Figure 1, panels C and D). DNA unwinding in the absence of ATP and $MgCl_2$ can be ascribed to RP-A since the peak of activity coincides (i) with the three RP-A subunits of 70, 32 and 11 kDa, respectively (Figure 1E, a 55 kDa band always seen in bovine RP-A is a proteolytic degradation product (35). (ii) These bands all reacted with antibodies raized against RP-A (data not shown) and (iii) DNA polymerase δ in the presence of PCNA and RF-C was stimulated by the same fractions on primed M13 DNA (Figure 1B). The separated DNA helicase, named DNA helicase F, was free of RP-A and did not stimulate DNA polymerase δ (Figure 1B).

Biochemical characterization of calf thymus DNA helicase F

The unwinding activity depending on ATP and $MgCl_2$ was further purified on a FPLC Superose gel filtration column. Two peaks of DNA helicase activity were eluted, one at the position of bovine serum albumin (M_r 67 kDa) and the other near lactate dehydrogenase (M_r 142 kDa), suggesting that the enzyme tends to oligomerize (Figure 2). Next we performed photo-affinity labeling of the DNA helicase and the results shown in Figure 3 indicated that a band exclusively at 72 kDa was radioactively labeled with [α^{32}]ATP. Thus we conclude that the DNA helicase F exists as polypeptide of 72 kDa. Table 1 summarizes data on the reaction optimum of DNA helicase F. The ATP optimum was 4 mM and that for $MgCl_2$ 5 mM. Calf thymus DNA helicase F is resistant to KCl up to 100 mM. The enzyme could use all four ribonucleoside-5′-triphosphates and all four deoxyribonucleoside-5′-triphosphates. ATP and dATP were however the favoured energy sources. Under these optimized reaction conditions (4 mM ATP, 5 mM $MgCl_2$ and 100 mM KCl) titration of DNA helicase resulted in saturation at a DNA helicase F to substrate ratio of 15 (Figure 4A) and the reaction kinetic performed with an enzyme to substrate ratio of 20. The reaction was linear for 60 min (Figure 4B), a common observation with many eucaryotic DNA helicases (1, 2)

Calf thymus DNA helicase F unwinds DNA in the 5′→3′ direction

Calf thymus DNA helicase F preferentially unwinds in the 5′→3′ direction as indicated by the data shown in Figure 5. In the same experiment DNA helicase A, an enzyme unwinding in the 3′→5′ direction (6) was used as the 'control' enzyme. Titration of the unwinding direction showed saturation at 75 ng (Figure 5B) as observed for the optimized assay (Figure 4A). The extent of the

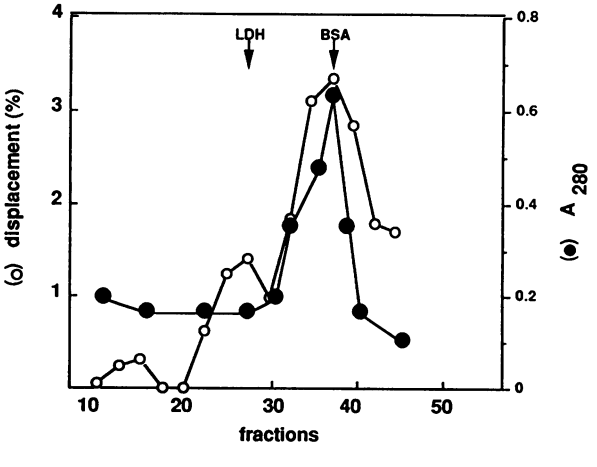

Figure 2. Gel filtration of calf thymus DNA helicase F on a FPLC-Superose column. 100 μg of DNA helicase F from the FPLC- Mono Q column (fractions 49−59, from Figure 1) were chromatographed as described in Materials and Methods. Each second fraction was tested for DNA helicase activity (open circles) and protein concentration (closed cicles). BSA: bovine serum albumin ; LDH: lactate dehydrogenase.

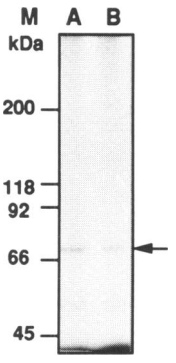

Figure 3. Affinity labeling calf thymus DNA helicase F. Affinity labelling was performed with 700ng (lane **A**), 350ng (lane **B**) of DNA helicase F (Mono-Q fraction 49). The reaction products were resolved on a 10% SDS-PAGE as described in Materials and Methods.

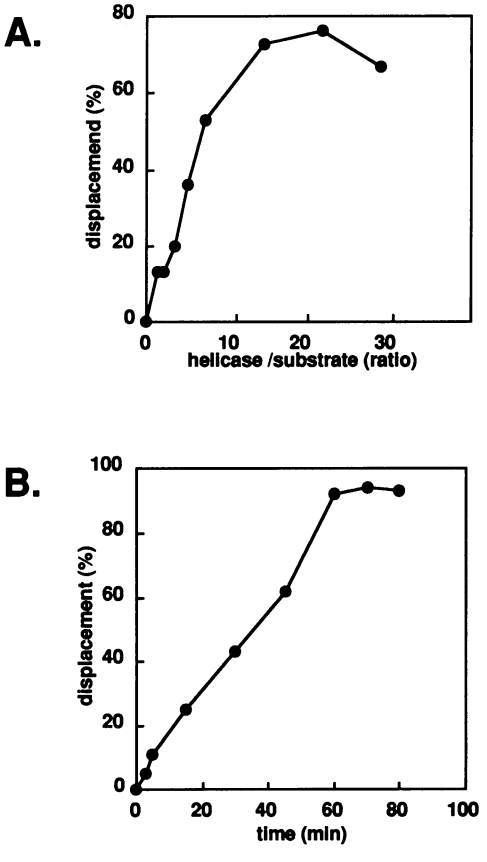

Figure 4. Titration and time course of calf thymus DNA helicase F on a 24-mer substrate. Titration of DNA helicase F was carried by using the DNA helicase assay described in Materials and Methods in the presence of 4 mM ATP, 5 mM $MgCl_2$ and 100 mM KCl. The time course was obtained under the same condition by using a DNA helicase F (Mono-Q fraction 49)/ substrate ratio (w/w) of 20 per time point.

reaction was however only 65−70% on this directionality substrate. At highter concentrations of DNA helicase (>75 ng), the enzyme did also unwind the 3′→5′ nucleotide partially (20−30%), for which we do not have an explanation at this time.

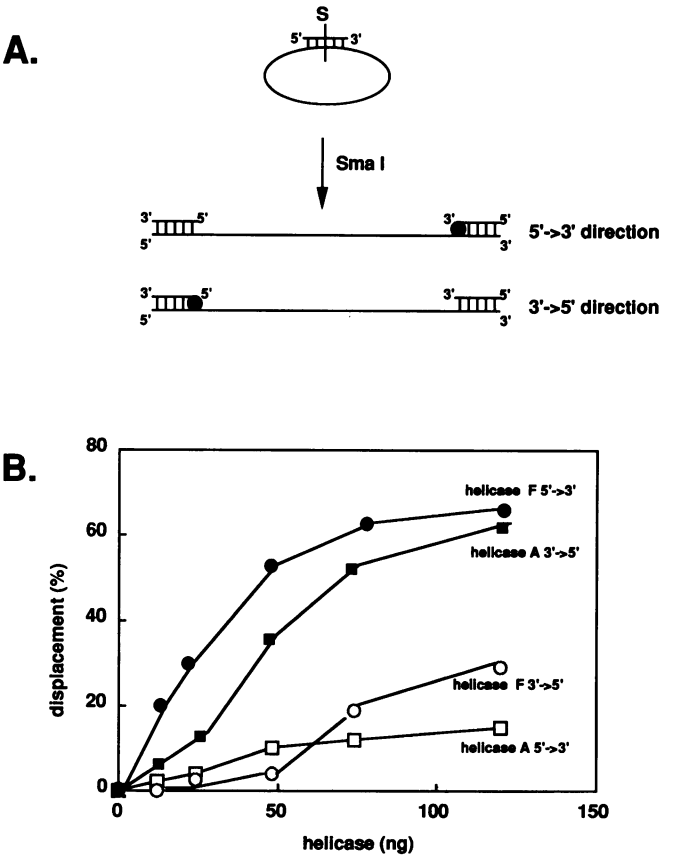

Figure 5. Calf thymus DNA helicase F unwinds DNA in the 5′→3′ direction. **A**: Directionality substrates. **B**: Titration of calf thymus DNA helicase A and DNA helicase F. Different amounts of DNA helicases A and DNA helicase F (Mono-Q fraction 49) were each titrated with the 5′→3′ and the 3′→5′ directionality substrates as described in Materials and Methods. Closed circles: DNA helicase F on the 5′→3′ directionality substrate; open circles: DNA helicase F on the 3′→5′ directionality substrate; closed squares: DNA helicase A on the 3′→5′ directionality substrate; open squares: DNA helicase A on the 5′→3′ directionality substrate.

Substrate requirements of calf thymus DNA helicase F

DNA helicase F was tested on a variety of different DNA substrates (Figure 6A). A 17-mer oligonucleotide was displaced to 90% and this was also evident if the substrate contained a ss tail of 15 nucleotides either at the 3′ end (98%) or at the 5′ end (91%) or on both ends (95%). When the length of the double stranded region was increased a rapid decrease of activity was evident (compare panels d, e and f). These data were plotted in Figure 6B and suggested that the critical length (50 % displacement remaining) under these conditions were about 20−25 nucleotides. A blunt end substrate did not serve as a substrate for calf thymus DNA helicase F (panel h), neither did a variety of constructed oligonucleotides containing ss regions of 42 to 84 nucleotides on either side of a double stranded region with a length of 15 to 17 nucleotides. From the 5′→3′ directionality one would predict that substrates i, k and l could serve as displacement substrates. Since they could not, we conclude that calf thymus DNA helicase F requires ss region longer than 84 bases to be loaded onto DNA.

Table 1. Properties of calf thymus DNA helicase F[1]

Component: ATP mM	unwinding (%)	$MgCl_2$ mM	unwinding (%)	KCl mM	unwinding (%)	Nucleotides[2] mM	unwinding (%)
0	3	0	15	0	75	ATP	84
0.25	35	0.5	81	20	80	CTP	58
0.5	50	1	86	40	91	UTP	62
1	65	3	89	100	92	GTP	57
2	73	5	90	200	30	dATP	83
4	85	10	85	300	10	dCTP	67
6	60					dTTP	46
10	57					dGTP	57

[1]DNA helicase (Mono-Q fraction 49) was tested as described in Materials and Methods by varying the concentrations of ATP, $MgCl_2$, KCl and the eight nucleoside or deoxynucleoside -5'-triphosphate. When components were tested the other components were at the following concentrations: ATP: 1 mM; $MgCl_2$: 1 mM; KCl: 0 mM.
[2]each at 1 mM.

Calf thymus RP-A enables its copurifying DNA helicase F to unwind long DNA substrate

We have found earlier that calf thymus DNA helicases A, B, C and D can be stimulated by calf thymus RP-A but not by SSB from *Escherichia coli* and its bacteriophage T4 counterpart (9). Furthermore this stimulation was species specific since the RP-A from human cells was unable to do so. We therefore tested the effect of calf thymus RP-A to stimulate the homologous DNA helicase F on a substrate with a long double stranded region of up to 400 nucleotides. DNA helicase F behaved in a very similar way as the four DNA helicases A, B, C and D (9) since neither *Escherichia coli* nor the bacteriophage T4 SSB did stimulate unwinding (data not shown). Titration of stimulation was carried out up to 250 ng in the presence or absence of DNA helicase F, and as expected from earlier observations (30) RP-A could unwind DNA above 150 ng (in the absence of ATP and $MgCl_2$). Thus an amount of 100 ng was included to carry out a kinetic experiment. Figure 7 shows that RP-A can enable the DNA helicase F to unwind long double stranded regions. The degree of stimulation was similar from 5 to 90 min suggesting that RP-A may either prevent reannealing or renders the DNA helicase F more processive.

DISCUSSION

Upon isolation of RP-A a DNA helicase extensively copurified up to the last step of purification (30) and could be separated on a FPLC Mono Q column (Figure 1). This DNA helicase shares some biochemical properties with calf thymus DNA helicases B and C (9). A similar DNA helicase has been described from HeLa cell and is called DNA hellicase IV (13). Both enzymes have the same 5'→3' directionality, can only unwind very short DNA fragments, require very long ss region (>84 bases) to bind, and have a similar enzyme to substrate ratio. Finally a complex between calf thymus RP-A and a DNA helicase was found to stimulate DNA polymerase α on double stranded DNA (36). In sum, our data indicated that the enzyme described in this paper has not been identified in calf thymus and we tentatively name it as DNA helicase F.

DNA helicase F does not depend on a fork-like structure (Figure 6), but the substrate tested might not reflect the situation at the replication fork. On the other hand the fact that a very long ss DNA region is required for the enzyme to bind is suggestive of a role at the replication fork. Another hint for a possible function at the replication fork is the fact that strand displacement by DNA polymerase δ in the presence of RP-A is stimulated by the DNA helicase F (B. Sturzenegger and U. Hübscher, unpublished data). It is less probable that the DNA helicase F is involved in DNA repair and transcription since the long ss region to be loaded onto the DNA are not part of an initial step of these processes.

Very little is known about the *in vivo* roles of cellular DNA helicases described so far (reviewed in ref. 2). For example, DNA helicase A, B, C and D (6) and the DNA helicase F (Figure 7) from calf thymus as well as a RP-A dependent DNA helicase from human cells (14) are stimulated by RP-A to unwind long stretches of DNA. In case of the calf thymus enzyme this stimulation was species-specific since it was only evident with homologous RP-A but not with the corresponding proteins from prokaryotes and human (9, 37). However, although RP-A was originally found to be an essential auxiliary protein during the replication of SV40 DNA, it was subsequently found to play a role also during recombination (38) and repair (39) processes so that these helicases still could be involved in either of these functions.

Recent progress in elucidating the function of DNA helicases came from yeast *Saccharomyces cerevisiae* where biochemical characterization of the Rad3 DNA helicase identified a clear role in DNA repair (40, 41). The function of another yeast DNA helicase, called SRS2, appears to be in the error-prone repair pathway and in recombination (20). Recent data suggested that a DNA repair helicase is a component of BTF2 (TFIIH) basic transcription factor resulting in a connection between transcription and a DNA helicase (21). Finally, some human Xeroderma pigmentosum and rodent excision reapir cross complementing (ERCC) genes appear to be DNA helicases, since it was found that the yeast RAD3 DNA helicase is homologous to XP-D and to ERCC2 and RAD25(SSL2) to XP-B and ERCC3, respectively (42). In addition further yeast DNA helicases have been identified (43) one of which is likely associated with replication factor C (22), an auxiliary protein of DNA polymerases δ and ϵ, and the other is called DNA helicase III with so far no clear biological function (23).

Since viral model systems are genetically more amenable, the properties of viral DNA helicases are known in more detail. Thereof the SV40 T antigen helicase is the best characterized example (reviewed in ref. 24). The T antigen binds to the core

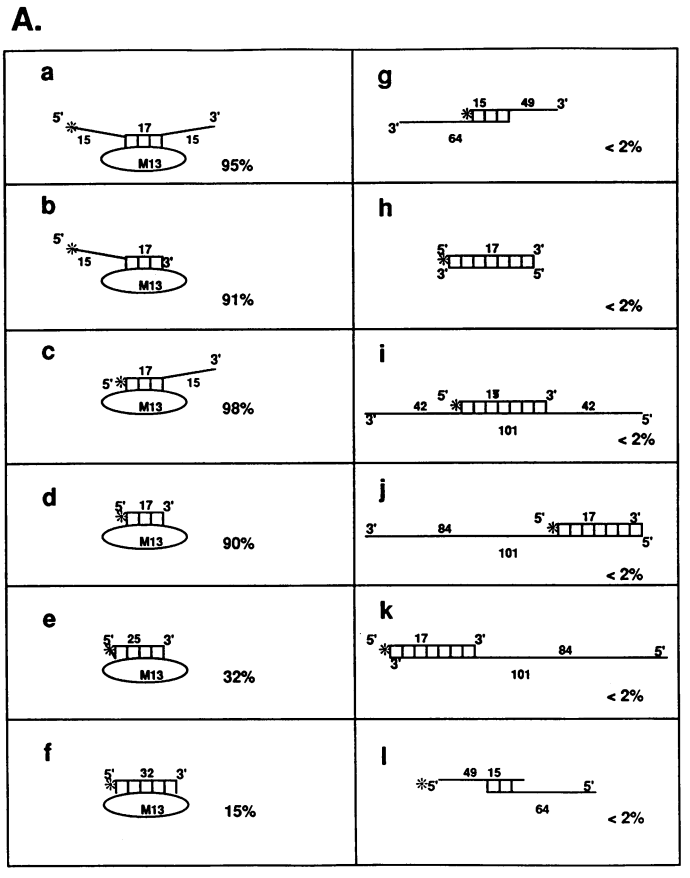

Figure 6. Substrate requirement of calf thymus DNA helicase F. DNA helicase assay conditions were as described in Materials and Methods in the presence of 4 mM ATP, 5 mM $MgCl_2$ and 100 mM KCl by using saturating amounts of calf thymus DNA helicase F (Mono-Q fraction 49, 100 ng) as determined in Figure 4. The numbers given in each box represent the % displacement as compared to the substrate in the absence of enzyme (representing 0%) and to the heated substate (representing 100%). **A** (panels a–e): Test of 12 different substrates; **B**: Blot of panel d, e and f from A.

origin of replication in the presence of ATP and induces local unwinding in the vicinity. The T-antigen oligomerizes in the presence of ATP to two hexamers. DNA unwinding by T antigen helicase now starts in the presence of ATP and another protein

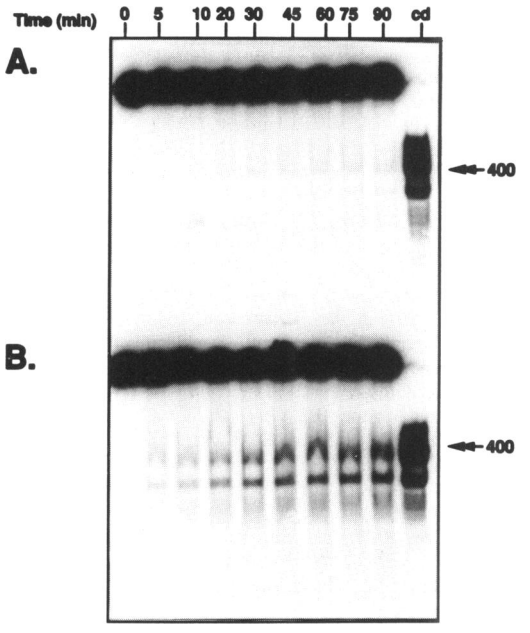

Figure 7. Calf thymus RP-A enables calf thymus DNA helicase F to unwind a long DNA substrate. Kinetic experiments were carried out under conditions descibed in Figure 4B by using a substrate with an annealed fragment of about 400 bases. **A**: Calf thymus DNA helicase F alone (Mono-Q fraction 49, 75 ng); **B**: Calf thymus DNA helicase F (Mono-Q fraction 49, 75 ng) in the presence of 100 ng calf thymus RP-A.

called RP-A (44). In Herpes Simplex Virus type 1 the DNA helicases are functionally well characterized (rewieved in (25)). The UL9 DNA helicase binds to the origin of replication where it acts as an initiator protein (45). At the Herpes Simplex Virus type 1 replication fork itself another DNA helicase is required. It exists as a heterotrimeric protein (UL5, UL8 and UL52) that has the two enzymatic activities of primase and helicase (46). The interesting notion is that the respective single subunits do have neither primase nor helicase activities. At least a heterodimer of UL5 and UL52 has to be formed in order to be active as helicase and primase (47). Finally replication of the bovine papilloma virus requires two proteins called E1 and E2 (rewieved in ref. 48). Thereof E1 was identified as an origin binding DNA helicase (26, 27).

Like in *Escherichia coli* (1) DNA helicases are now identified in eukaryotes in increasing number. We must know the corresponding genes in order to learn: (i) how they are organized, (ii) if they are essential for the viability of the cell, (iii) if they are regulated throughout the cell cycle, and (iv) how they can possibly act at model replication forks.

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