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Mammalian DNA helicase

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ABSTRACT

A forked DNA was constructed to serve as a substrate for DNA helicases. It contains features closely resembling a natural replication fork. The DNA was prepared in large amounts and was used to assay displacement activity during isolation from calf thymus DNA polymerases α holoenzyme. One form of DNA polymerase α holoenzyme is possibly involved leading strand replication at the replication fork and possesses DNA dependent ATPase activity (Ottiger, H.-P. and Hübscher, U. (1984) Proc. Natl. Acad. Sci. USA 81, 3993-3997). The enzyme can be separated from DNA polymerase α by velocity sedimentation in conditions of very low ionic strength and then be purified by chromatography on Sephacryl S-200 and ATP-agarose. At all stages of purification, DNA dependent ATPase and displacement activity profiles were virtually superimposable. The DNA dependent ATPase can displace a hybridized DNA fragment with a short single-stranded tail at its 3'hydroxyl end only in the presence of ATP, and this displacement relies on ATP hydrolysis. Furthermore, homogeneous single-stranded binding proteins from calf thymus as well as from other tissues cannot perform this displacement reaction. By all this token the DNA dependent ATPase appears to be a DNA helicase. It is suggested that this DNA helicase might act in concert with DNA polymerase α at the leading strand, possibly pushing the replication fork ahead of the polymerase.

INTRODUCTION

DNA replication depends on the coordinated activities of many enzymes and accessory proteins (1,2). Among them, a group of proteins is responsible for maintaining and transiently abolishing the helical structure of DNA (3). Opening of the double helix in advance of DNA replication is necessary for efficient and processive polymerization of the daughter strands (1,2). This strand separation is performed by enzymes called DNA helicases. They are capable of using the energy of nucleoside triphosphate hydrolysis to actively unwind the double-stranded DNA (4). So far, unequivocal demonstration of DNA helicases has been accomplished in prokaryotes only. Four DNA helicases (called I,II,III and rep) have been studied in detail in Escherichia coli (reviewed in 5). Analogous enzymes are the gene 4 protein from bacteriophage T7 (6), the gene 41 protein (7,8) and the dda protein (9,10) from

bacteriophage T4. Furthermore, several additional DNA dependent ATPases, such as DNA gyrase, dnaB protein, protein n', recA protein, recBC nuclease or an ATPase associated with DNA polymerase III holoenzyme (1,2,11) were found, the latter all have important functions in DNA replication, recombination or repair.

In eukaryotes, many reports of different DNA dependent ATPases have appeared in the last few years (12-22). Some of them have been purified to apparent homogeneity and partially characterized biochemically (16,17, 20-22). Their occurrence in multiple forms is suggestive of distinct functional roles in DNA metabolism (20,22) but, owing to the lack of conditional lethal mutants and of functional assays, little is known about their actual in vivo tasks.

In the course of our studies with calf thymus DNA polymerase α holoenzyme (23) we identified a DNA dependent ATPase associated with one holoenzyme form (24). We now present evidence that this enzyme is a DNA helicase.

MATERIALS AND METHODS

Chemicals.

ATP, unlabeled dNTP's and ATP-agarose were from P-L Biochemicals, and the purity of the ATP was tested by high performance liquid chromatography. [8-^{3}H]ATP, [^{3}H]dTTP, [^{3}H]dATP and [α^{32}P]dTTP were purchased from Amersham. Adenosine 5'-O-(3-thiotriphosphate) was obtained from Boehringer. Pharmacia and Miles provided Sephacryl S-200 and bovine serum albumin, respectively. Sodium bisulfite and ammonium sulfate were from British Drug House, trypsin from Worthington, dithiothreitol from Calbiochem, pepstatin from Sigma, and N-ethylmaleimide from Serva.

Nucleic acids.

Single stranded (ss) DNA from minute virus of mice, a parvovirus, was donated by R. Sahli (ISREC Lausanne), and DNA from phage M13(mp8) was isolated according to ref. 25. Priming of ssM13 with the pentadecamer oligodeoxynucleotide 5'-T-C-C-C-A-G-T-C-A-C-G-A-C-G-T-3' (New England Nuclear) and activation of calf thymus DNA were performed as described (24). P-L Biochemicals provided poly(dT) and the oligonucleotides $(dT)_{15}$, $(dT)_{20}$ and $(dT)_{25-30}$.

Enzymes and Proteins.

Escherichia coli single-stranded binding protein (ssB) was purified according to 26 and an SSB from calf thymus (UP1) according to 27. The high mobility group protein HMG1 was isolated under physiological conditions as described (28). DNA polymerase α holoenzyme (purified according to 23) was a gift from

M. Hässig (this institute). The two homogeneous DNA helicases T4 dda helicase and rep protein were generous gifts from Dr. C.V. Jongeneel (ISREC, Lausanne) and Dr. N. Arai (Palo Alto), respectively. Homogeneous Hela DNA topoisomerase I and Escherichia coli DNA gyrase were kindly donated by Dr. T. Pedrini (Pavia) and Dr. R.L. Low (St. Louis). Terminal deoxyribonucleotidyltransferase was purchased from Stehelin AG., Basel.

Preparation of the helicase substrate.

Pentadecamer primer (8 nmol) was oligo dT-tailed with terminal deoxynucleotidyltransferase (15 units) by incubation at 37°C in a final volume of 115 µl containing the following ingredients. 200 mM potassium cacodylate, pH 7.2, 40 mM KCl, 1 mM $CoCl_2$, 60 µM [α-^{32}P]dTTP (25,000 - 30,000 cpm/pmol), and 0.5 mM dithiothreitol. In a preliminary experiment, aliquots of 20 µl were removed after various time intervals and the lengths of the products analyzed on a Sephadex G-75 column (10 x 120 mm) previously calibrated with oligo $(dT)_{15}$, oligo $(dT)_{20}$, oligo $(dT)_{25-30}$ and poly dT. Chromatography was performed in 10 mM Tris·HCl, pH 7.5, 1 mM EDTA and 30 mM NaCl. Products with the size of about 30 bases were then synthesized in larger amounts and, after termination of the reaction with 10 mM EDTA, hybridized at a ratio of 1:400 (wt/wt) to ssM13(mp8) DNA. After incubation at 55° C for 5 min the DNA was slowly cooled to room temperature. Then the DNA was deproteinized by treatment with proteinase K (100 µg/ml) and 1% (wt/vol) $NaDodSO_4$ for 1 h at 37°C. The DNA was precipitated in 0.3 M NaOAc, pH 5.5, with 70% ethanol. After centrifugation (10,000 x g, 15 min) the precipitate was resuspended in 50 µl 10 mM Tris·HCl, pH 7.5, 1 mM EDTA, and the primed M13 DNA purified by velocity sedimentation in a 10%-30% (wt/vol) sucrose gradient in 20 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1M NaCl. Centrifugation was performed in a Sorvall TST 60.4 rotor at 38,000 rpm for 4 hr at 4°C. Fractions (150 µl) were collected from the bottom of the tube and the radioactivity of each determined. The radioactive fractions from the bottom half of the tubes were pooled. The purified substrate was free of non-hybridyzed oligonucleotides and contained only a trace amount of unincorporated [α-^{32}P]dTTP (see Figure 1).

Enzyme assays.

(i) DNA dependent ATPase. The final volume of 10µl contained. 20 mM Tris·HCl, pH 7.5, 4% (wt/vol) sucrose, 8 mM dithiothreitol, 80 µg/ml bovine serum albumin, 10 mM KCl, 1 mM $MgCl_2$, 1 mM [8-^{3}H]ATP (10-20 cpm/pmol), 500 pmol ssM13 DNA (as nucleotides), and enzyme to be assayed. Incubation was at 37°C for 30 min unless otherwise mentioned. Two-µl aliquots were spotted onto a PEI thin-layer plate, and one-µl of a marker solution containing 25 mM each of ATP

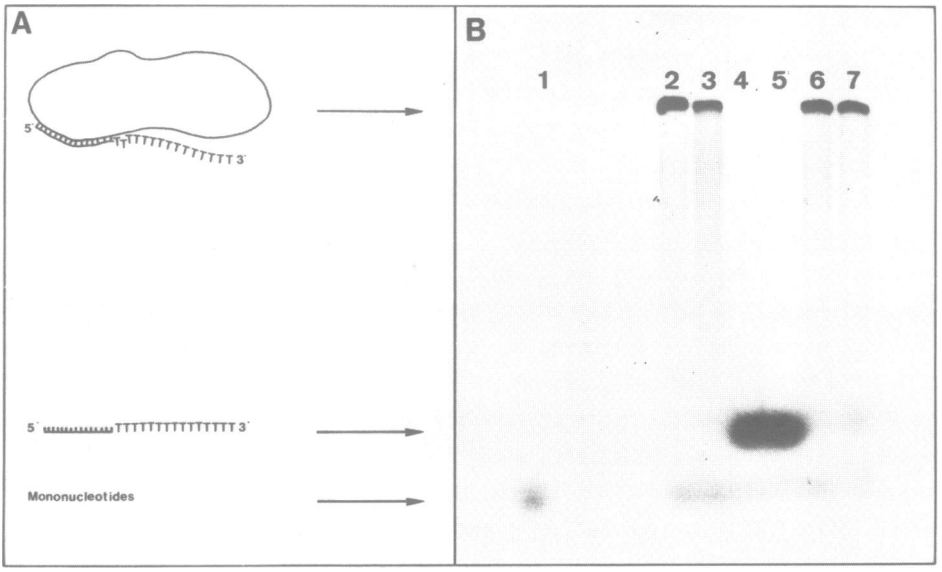

Figure 1. DNA helicase assay. (A): Educt and products of the DNA helicase assay. A pentadecamer containing a 10-15 base long radioactive dT-tail at its 3'-hydroxyl end is hybridized to ssM13(mp8) DNA. This substrate is incubated with the sample containing helicase. The enzyme displaces the oligodeoxynucleotide fragment from the ssDNA circle, and the products are separated by electrophoresis in a 6% polyacrylamide gel. Primed ssM13 DNA does not enter the gel, the displaced fragment migrates half-way through the gel, and mononucleotides run to the front. (B): Autoradiographic demonstration of the DNA displacement reaction. Lane 1: [α-^{32}P]dTTP alone (1000 cpm), lane 2: DNA substrate alone, no enzyme, lane 3: substrate and DNA polymerase α holoenzyme containing the DNA dependent ATPase activity (10 units DNA dependent ATPase), no ATP, lane 4: as lane 3 but with ATP (1 mM), lane 5: the oligo·(dT) elongated, non hybridized oligodeoxynucleotide alone, lane 6: as lane 4, but with the enzyme heated to 90°C for 3 min, lane 7: as lane 3, but with the ATP replaced by the non hydrolyzable ATP analog adenosine 5'-O-(3-thiotriphosphate) (1 mM).

and ADP was applied on top of it. ATP and ADP were separated by chromatography in 0.5 M LiCl and 1 M formic acid. The strip was inspected under UV-light and the regions containing ATP and ADP were cut out and counted in liquid scintillation fluid. One unit of eukaryotic DNA dependent ATPase is defined as the activity that cleaves 1 nmol ATP per 30 min at 37°C (24) and one unit of prokaryotic DNA dependent ATPase as 1 µmol ATP per min at 37°C (10).

(ii) DNA helicase assay. The final volume of 50 µl contained 20 mM Tris·HCl, pH 7.5, 4% (wt/vol) sucrose, 8 mM dithiothreitol, 80 µg/ml bovine serum albumin, 10 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 3000 - 5000 cpm [^{32}P]-labelled M13 helicase substrate 2-3x10^5 cpm/µg), and enzyme to be assayed. Incubation was

at 37°C for 30 min unless otherwise mentioned. The reaction was stopped by bringing the solution to a final concentration of 1% wt/vol $NaDodSO_4$, 20 mM EDTA, 5% (vol/vol) glycerol, and 0.15 mg/ml bromophenol blue. After further incubation at 37°C for 5 min the sample was loaded onto a 6% (w/v) polyacrylamide gel (14 x 11 x 0.15 cm) containing 40 mM Tris·HCl, pH 7.9, 20 mM NaOAC, and 1 mM EDTA. Electrophoresis was carried out at 100 V for 150 min, the gel dried under vacuum and exposed to a Kodak X-omat film for autoradiography. The primed, radioactively labeled M13 DNA does not enter the gel, the displaced oligodeoxynucleotide fragment (25-35 bases long) migrates half-way through the gel and residual, unincorporated mononucleotides run to the front. For exact quantification, the gel area corresponding to the displaced fragment was cut out and counted in liquid scintillation fluid. One unit of DNA helicase is defined as the activity that displaces 1 pmol of oligodeoxynucleotide fragments (as nucleotides) per 30 min at 37°C.

(iii) DNA polymerase. DNA polymerase α activity on activated DNA, and DNA polymerase α holoenzyme activities on primed M13 DNA or ss parvoviral DNA were determined exactly as described (24).

Copurification of DNA dependent ATPase and DNA helicase.

The DNA polymerase α holoenzyme containing DNA dependent ATPase activity was purified by ammonium sulfate precipitation, phosphocellulose- and DEAE-cellulose chromotography exactly as described (23,24). Form A eluted from the DEAE-cellulose column contains the DNA dependent ATPase (24). The ATPase was separated from the DNA polymerase α by velocity sedimentation in a 20-40% (vol/vol) glycerol gradient containing low salt (see legend to Figure 2 for details). DNA dependent ATPase (10,000 units) from six pooled gradients were applied to a Sephacryl S-200 column (2.5x75cm) in buffer A (20 mM potassium phosphate, pH 7.5, 40 mM KCl, 0.1 mM EDTA, 1 mM 2- mercaptoethanol, 1 µM pepstatin, and 10 mM $NaHSO_3$). Gel filtration was performed at a flow rate of 30 ml/hr. Fractions (5 ml) were collected and assayed for DNA dependent ATPase and for DNA helicase activity. The peak fractions (50% of the total activity) were pooled and directly applied onto an ATP-agarose column (0.5 x 3 cm) in buffer A. After washing the column with 2 ml buffer A, a 10 ml ATP gradient (0 - 10 mM) in buffer A was applied. Fractions (0.2 ml) were collected and tested for DNA dependent ATPase and DNA helicase activity. Both activities showed virtually superimposable elution profiles. The peak fractions were pooled, dialysed against buffer A, divided into aliquots, frozen in liquid nitrogen, and stored at -80°C. The purified DNA dependent ATPase (12,500 units ATPase / mg) was enriched over 5000-fold in the final preparation compared to the crude extract, with a total recovery of 2.5%.

Other Methods.

Determinations of DNA topoisomerase I and DNA gyrase were as described (24). Protein determination was according to (29) using bovine serum albumin as a standard.

RESULTS

DNA helicase assay.

The ssM13(mp8) DNA, when primed with the pentadecamer 5'-T-C-C-C-A-G-T-C-A-C-G-A-C-G-T-3,' contains dA residues at position 1,3,18,26, and 27 on the 3' side of the pentadecamer primer (30). Elongation of the primer at the 3' terminus for 10-15 dT residues using terminal deoxynucleotidyltransferase and [α^{32}P]dTTP, creates an unpaired 3'-tail after the first residue. The latter mimicks the leading strand of a replication fork, and can therefore be assumed to be a quasi natural substrate for helicases. These will be identified by their ability to displace the radioactive, elongated primer.

Figure 1 shows an example of the DNA displacement reaction. The DNA polymerase α holoenzyme containing DNA dependent ATPase activity (form A from ref. 24) displaces a fragment (lane 4) that migrates with the same mobility as the unpaired, elongated primer (lane 5). The displacement activity is heat labile (lane 6) and dependent on ATP (lane 3). Displacement is prevented by more than 95% if ATP is replaced by the non-hydrolyzable ATP analog adenosine 5'-O-(3-thiotriphosphate) (lane 7). Both the helicase and the ATPase assays were used to monitor the further purification of the enzyme.

Cosedimentation of the DNA dependent ATPase and helicase activities under conditions where both were separated from DNA polymerase α.

The DNA dependent ATPase is associated with the form A DNA polymerase α holoenzyme (24) and cannot be separated from the DNA polymerase at physiological or very high ionic strength (>625 mOsm, 24). If, however, velocity sedimentation is performed at very low ionic strength (90 mOsm) the DNA polymerase and the ATPase separate. At the same time cosedimentation of ATPase and displacement activity are maintained (Fig. 2A,B).

The purified enzyme has both DNA dependent ATPase and DNA helicase activity.

The DNA dependent ATPase separated from the DNA polymerase α by velocity sedimentation (Figure 2A) was purified further and completely separated from residual DNA polymerase α by gel filtration on Sephacryl S-200 and by affinity chromatography on ATP-agarose (see Materials and Methods). At all steps, the elution profiles corresponding to DNA-dependent ATPase and DNA helicase

activity were, virtually superimposable. Reaction requirements for helicase activities are shown in Table 1. The displacement of the fragment was dependent on ATP as well as $MgCl_2$. Replacement of ATP by the non-hydrolyzable analog adenosine-5'-O-(3-thiotriphosphate) prevented helicase action. Heat and protease treatment destroyed the enzymatic activity. Finally, N-ethylmaleimide blocked the enzymatic activity indicating an essential sulfhydryl group. Electrophoresis of the reaction product in a 12% polyacrylamide -7M urea gel showed that the displaced fragment maintained the original length of 25-35 bases (data not shown).

Kinetics of DNA helicase.

DNA helicase activity is proportional to the amount of enzyme added (Figure 3A). Using a saturating amount of enzyme the rate of displacement is linear for at least 15 min (Figure 3B). The displacement rate is reduced more than 50-fold if no ATP is included in the reaction.

TABLE 1 Reaction requirements of the purified DNA helicase [a]

Reaction conditions	Displacement (pmol) [b]
Complete [c]	24.1
- Enzyme	<1
- $MgCl_2$	1
+ EDTA (10 mM)	<1
- ATP	2.5
- ATP, + adenosine 5'-O-(3-thiotriphosphate) (1 mM)	1.8
+ N-ethylmaleimide (10 mM)	3.2
+ NaCl (130 mM)	5.2
+ Ammoniumsulfate (45 mM)	8.1
+ KCl (130 mM)	2.3
- Enzyme, + heated Enzyme [d]	<1
- Enzyme, + trypsin-treated Enzyme [d]	<1

a Enzyme source was the DNA dependent ATPase/DNA helicase preparation from the ATP-agarose column (see Materials and Methods).
b Calculated by determination of the radioactivity of the displaced fragment separated by gel electrophoresis as described in Materials and Methods.
c The displacement assay was carried out as described in Materials and Methods.
d The purified helicase (20 units) was heated for 5 min at 90°C or digested with 1 unit of trypsin for 1 h at 37°C.

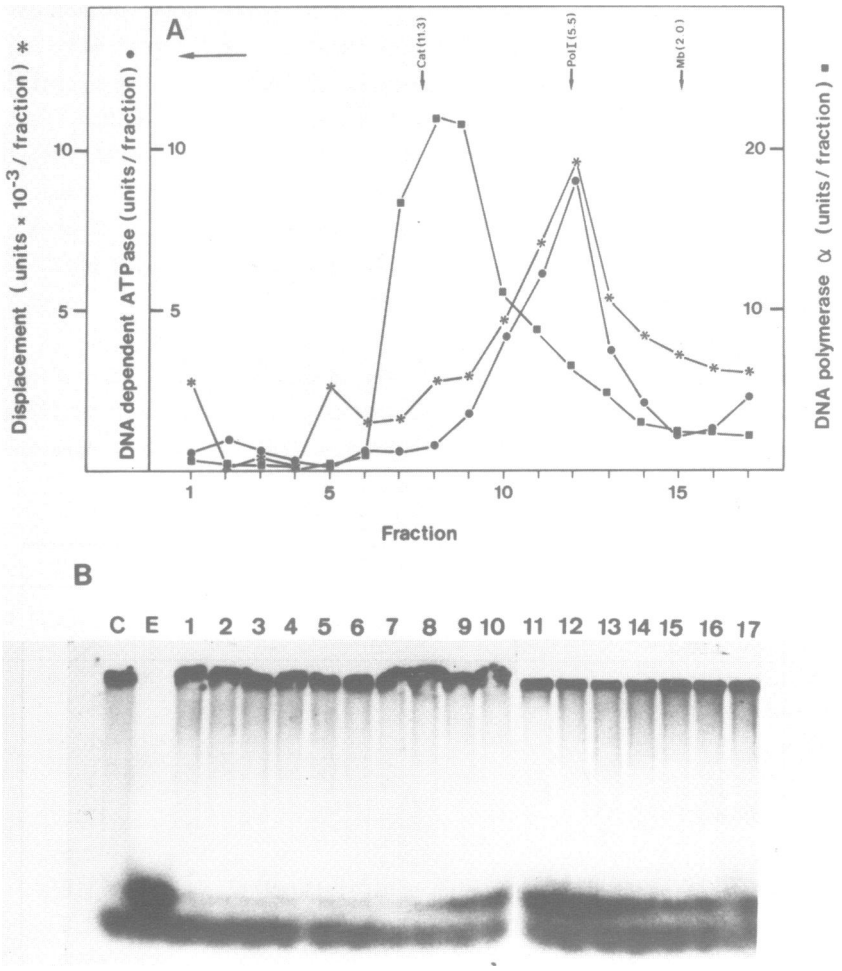

Figure 2. Velocity sedimentation of DNA polymerase α holoenzyme, form A, under low salt conditions. 200 units of DNA polymerase α (peak A from the DEAE cellulose column, (ref. 24) were dialyzed against 20 mM potassium phosphate, pH 7.5, 0.1 mM EDTA, 1 mM dithiothreitol, 1 μM pepstatin and 40 mM KCl. The sample was loaded onto a preformed 3.6 ml linear 20-40% (vol/vol) glycerol gradient in the same buffer and centrifugated in a Sorvall TST 60.4 rotor at 44,000 rpm at 4°C for 20 h. Gradient fractions (180 μl) were collected from the bottom.
Individual fractions were tested for (i) DNA polymerase α holoenzyme using primed M13 DNA as the template, (ii) DNA dependent ATPase using ssM13 DNA as the cofactor, and (iii) the displacement helicase activity as described in Materials and Methods. Sedimentation standards were run in a parallel tube and detected as follows. myoglobin (Mb) at A_{430}, DNA polymerase I (Pol I) as described in Materials and Methods for DNA polymerase α, and catalase (Cat) as

described in the Worthington manual. Arrow indicates direction of sedimention. (A) □, DNA polymerase α, ●, DNA dependent ATPase, * displacement helicase activity. (B) Autoradiogram of an electrophoresis gel showing the radioactive product of the displacement helicase reaction. The numbers correspond to the gradient fractions shown in (A), C refers to a control with substrate incubated in gradient buffer without enzyme, and E is the dialyzed enzyme (1 µl) prior to loading onto the gradient. For each gradient fraction, the area corresponding to the displaced, radioactive fragment was cut out and quantitated in a liquid scintillation counter, the data were used to construct the displacement profile in (A).

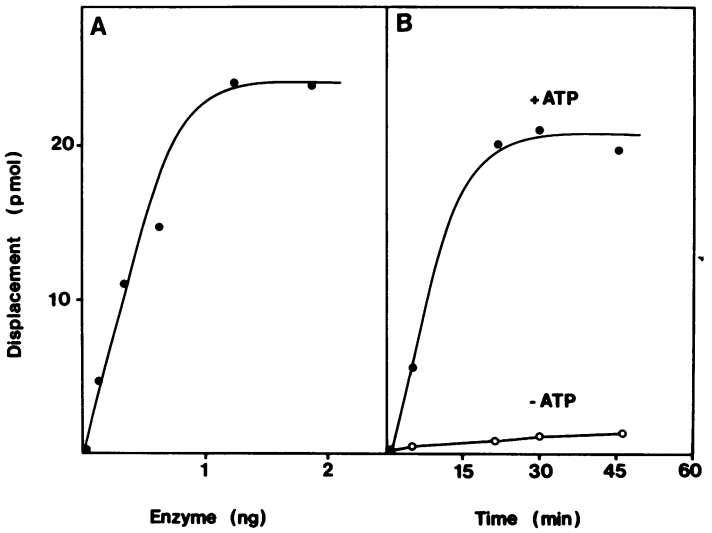

Figure 3. Kinetics of DNA helicase action. (A) Increasing amounts of the purified DNA helicase were incubated for 30 min in the displacement reaction, the products electrophoresed and quantitated by liquid scintillation counting as described in Materials and Methods. (B) A saturating amount (1 ng) of enzyme was used to monitor the time course of the reaction in the presence and absence of ATP (1 mM).

Prokaryotic DNA helicases can, but several other DNA binding proteins cannot perform the displacement reaction.

The well characterized prokaryotic helicases Escherichia coli rep protein (31,32) and T4 dda helicase (9,10) can displace the labeled fragment (Table 2), and this displacement is ATP dependent (data not shown). On the other hand, several putative unwinding proteins, such as the E. coli SSB (26), the calf thymus unwinding protein UP1 (27) or the calf thymus HMG1 (28) were unable to perform this displacement. DNA topoisomerases of type I and II were

both inactive in the DNA helicase assay. Finally, a developmentally regulated ssDNA binding protein from calf brain (33) could not carry out strand displacement.

DISCUSSION

The calf thymus DNA polymerase α holoenzyme can be separated into distinct forms with possible functions at the leading and lagging strand of the replication fork (24). The holoenzyme putatively assigned to the leading

TABLE 2 DNA helicase activity of various proteins [a]

Protein [b]	Displacement (pmol) [c]
Calf thymus DNA helicase [d]	27
Calf thymus DNA polymerase α holoenzyme, form A [e]	28
Calf thymus DNA polymerase α holoenzyme, form C [f]	2.5
T4 *dda* helicase	31
Escherichia coli rep helicase	29
Escherichia coli SSB	1.8
Calf thymus SSB(UP1)	<1
Calf thymus HMG1	1.2
Calf brain SSB [g]	1
Hela DNA topoisomerase I	<1
Escherichia coli DNA gyrase	3.4
Bovine serum albumin	<1

a DNA helicase assay was performed as described in Materials and Methods.

b The following amounts of enzymes were tested: DNA polymerases (1 unit), *dda* helicase (1 prokaryotic unit of DNA dependent ATPase), *rep* protein (1 prokaryotic unit of DNA dependent ATPase), topoisomerases (1 unit) and 1 μg each of *Escherichia coli* SSB, calf thymus SSB (UP1), calf thymus HMG1, calf brain SSB and bovine serum albumin.

c Calculated by determination of the radioactivity of the displaced fragment separated by gel electrophoresis as described in Materials and Methods.

d Enzyme source was the DNA dependent ATPase/DNA helicase preparation from the ATP-agarose column (see Materials and Methods).

e DNA polymerase α holoenzyme, form A (24)

f DNA polymerase α holoenzyme, form C (24) extensively purified according to (39).

g The calf brain SSB is a 35,000 dalton protein that is developmentally regulated (33).

strand contains an associated DNA dependent ATPase. The latter can be separated from the DNA polymerase α by velocity sedimentation in conditions of very low ionic strength (Figure 2). The DNA dependent ATPase, whether associated with the DNA polymerase α or resolved from it, appears to possess a DNA helicase activity for the following reasons: (i) The enzyme is able to perform strand displacement (Figures 1, 2 and 3), (ii) the displacement appears to be ATP dependent (Figure 1 and Table 1), (iii) the displacement relies on hydrolysis of ATP (Figure 1, Table 1), and (iv) the displacement activity cosediments with the DNA dependent ATPase on a glycerol gradient (Figure 2). It is unlikely that the enzyme is an enzymatic non-active single-stranded DNA binding protein since various putative helix destabilizing proteins such as Escherichia coli SSB (26), and calf thymus UP1 (27) or calf thymus HMG1 (34) could not carry out the strand displacement (Table 2).
Several DNA dependent ATPases have been isolated from eukaryotes in general (12-22) and from calf thymus in particular (35). Our DNA helicase ressembles to some extent the DNA dependent ATPase from Hela cells (21,36), because the Hela enzyme possesses an ATP dependent unwinding activity (36) and preliminary physico-chemical parameters of the DNA helicase, such as molecular weight (72,000), sedimentation coefficient (5.5-6.5) and preference for ssDNA over dsDNA (3 times) indicated similarities with the Hela enzyme (Hübscher, U. and Stalder, H.P., unpublished data).
Of the four E. coli DNA helicases (5) rep protein (31,32,37) and DNA helicase II (38) might separate strands at a replication fork in concert with DNA replication. Displacement of the DNA strands in connection with DNA replication is also performed by the bacteriophage proteins encoded by gene 4 of T 7 (6) and gene 41 of T 4 (7,8). The calf thymus DNA helicase might be involved in the advancement of the replication fork during replication. The enzyme is associated with DNA polymerase α holoenzyme (24) and can stimulate DNA polymerase α holoenzyme activity on a double stranded DNA template (Hübscher, U., unpublished data).
In conclusion, we have presented evidence for a mammalian DNA helicase. This enzyme is found associated with a form of DNA polymerase α (24). One might speculate at this time that it could be part of the "leading form of DNA polymerase α holoenzyme. The "lagging form" DNA polymerase α holoenzyme, in contrast, contains the primase (24). It will be interesting to learn whether a replication machinery working in vitro on a in vivo-like replication fork can be reconstituted from both types of holoenzymes, homologous SSB, and DNA topoisomerases.

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