We have purified a DNA helicase from calf thymus to apparent homogeneity by monitoring the activity with a strand displacement assay. DNA helicase followed the DNA polymerase α-primase complex through chromatography on phosphocellulose and hydroxylapatite. Separation from DNA polymerase α-primase complex as well as from the bulk of another DNA-dependent ATPase was achieved on heparin-Sepharose. Further purification steps included ATP-agarose and fast protein liquid chromatography-Mono S. A 47-kDa polypeptide cosedimented with the DNA helicase activity in a glycerol gradient as well as in gel filtration on Superose 6. The calf thymus DNA helicase had a sedimentation coefficient of 4–7 S and Stokes radius of about 45 Å suggesting that the enzyme might be monomer in its functional form.

DNA helicase activity requires a divalent cation with Mg²⁺ being more efficient than Mn²⁺ or Ca²⁺. Hydrolysis of ATP is required since the two nonhydrolyzable ATP analogues adenosine 5'-O-(3-thiotriphosphate) and adenylyl (β,γ-methylene)diphosphonate cannot substitute for ATP or dATP in the displacement reaction. Calf thymus DNA helicase is able to use ATP, dATP, dideoxy-ATP, CTP, and dCTP with Kₘ for ATP and dATP of 0.2 and 0.25 mM, respectively. The enzyme can displace a fragment of 24 bases completely in an enzyme concentration- and time-dependent manner. The DNA helicase appears to bind to single-stranded DNA and to move to single-strand double-strand transition. The directionality of unwinding is 3'→5' with respect to the single-stranded DNA to which the enzyme is bound.

DNA replication requires the concerted action of many enzymes and proteins (1, 2). Prior to DNA synthesis the double strands of the DNA helix must be melted to provide the DNA polymerases with single-stranded DNA. For this purpose enzymes called DNA helicases can transiently abolish the helical structure of the DNA (3). In general DNA helicases can hydrolyze nucleoside 5'-triphosphates in the presence of single-stranded DNA and appear to use the released energy to perform the unwinding of DNA (3). Unwinding of the genetic material is essential not only for chromosomal DNA replication (1, 2) but also for DNA repair processes such as mismatch (4) and excision repair (5). Finally, recombination (6), bacteriophage DNA replication (7), viral DNA replication (8), and bacterial conjugation (9) require the action of DNA helicases.

For this multiplicity of in vivo functions many different DNA helicases are now known in the bacterium Escherichia coli and its bacteriophages. Bacterial encoded DNA helicases include the rep protein (10), the dnaB protein (11), the primosomal protein n' (12), DNA helicase II (13) (known as the usc gene product (14)), DNA helicase III (15), and DNA helicase IV (16). DNA helicase I is the product of the tral gene of the F sex factor (17). The E. coli bacteriophage T7 contains its own DNA helicase, the gene 4 protein (18), and T4 has at least two helicases which are the gene 41 protein (19) and dda protein (20). The direction of unwinding can be either 5'→3' or 3'→5' along the single-stranded DNA where the enzyme moves. Rep, protein n', and helicase II and IV move in the 3'→5' direction, while the 5'→3' direction is performed by T7 helicase, the two T4 helicases, dnaB protein, helicase I, and helicase III.

In eukaryotes, on the other hand, only fragmentary information concerning different DNA helicases is currently available. The yeast Saccharomyces cerevisiae has so far two DNA helicases which are the rad3 gene product (21) and ATPase III (22). Rad3 helicase unwinds in the 5'→3' direction. However, the best characterized DNA helicase from eukaryotes is the SV40-encoded T-antigen, a multifunctional protein with DNA helicase activity (23, 24), working at the SV40 replication forks. Herpes simplex virus (type 1) encodes its own DNA helicase (25, 26). Finally, DNA helicases from chromosomal origin of higher eukaryotes were identified and so far only partially purified from calf thymus (27), mouse F93A cells (28), and Xenopus laevis oocytes (29).

In this paper we present the purification of a DNA helicase from calf thymus to apparent homogeneity after its separation from the DNA polymerase α-primase complex and another DNA-dependent ATPase. The purified enzyme consists of a 47-kDa protein, has low Kₘ for hydrolysis of nucleoside 5'-triphosphates, appears to bind to ssDNA, to move in a processive way to double-strand single-strand transition, and to unwind the DNA in the 3'→5' direction.

1 The abbreviations used are: ss, single-stranded; BSA, bovine serum albumin; FFLC, fast protein liquid chromatography; ATPγS, adenosine 5'-O-(3-thiotriphosphate); SDS, sodium dodecyl sulfate; dd, dideoxy; AMP-PCP, adenylyl-(β,γ-methylene) diphosphonate; BuPdGTP, N7-(p-n-butylyphenyl)-2-deoxyguanosine 5'-triphosphate.
**EXPERIMENTAL PROCEDURES**

**Materials**

Nucleotides, Column Supports, and Other Chemicals—Nucleoside 5′-triphosphates and deoxyribonucleoside 5′-triphosphates, heparin-Sepharose CL-6B, ATP-agarose (type 4), Superoxide 6 (prep grade), and Mono S (III 5/5) were purchased from Pharmacia LKB Biotechnology Inc. ATP-S and AMP-PCP were obtained from Boehringer Mannheim, and BuPDGT was the supplier of G.N. C. Worcester, MA. Amersham Corp. was the supplier for radioactively labeled nucleotides and deoxyribonucleoside triphosphates. Pepstatin and aphidicolin were obtained from Sigma, phosphocellulose from Whatman, and hydroxyapatite from Bio-Rad. All other reagents were of analytical grade and purchased from Merck (Darmstadt, FRG) or Fluka (Buchs, Switzerland).

Protein Nucleotides—Pharmacia LKB Biotechnology Inc. supplied the M13 universal sequencing primer (17-mer) and the synthetic polyaphidicolin were obtained from Sigma, phosphocellulose from Whatman, and hydroxyapatite from Bio-Rad. All other reagents were of analytical grade and purchased from Merck (Darmstadt, FRG) or Fluka (Buchs, Switzerland).

Nucleic Acids—Pharmacia LKB Biotechnology Inc. supplied the M13 universal sequencing primer (17-mer) and the synthetic poly-nucleotides poly(dA) and oligo(dT12-18). ssM13mp9 and M13mp11 DNA were prepared according to Maniatis et al. (30). Both substrates were cut with SmaI and annealed to ssM13 (mpg) DNA (1 µg) in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM MgCl₂, 1 µM pepstatin, 0.2 mM phenylmethylsulfonyl fluoride; buffer B, 20 mM potassium phosphate (pH 7.5), 10% (v/v) glycerol, 1 mM dithiothreitol, 4 mM MgCl₂, 1 µM pepstatin, 0.2 mM phenylmethylsulfonyl fluoride; buffer C, 20 mM potassium phosphate (pH 7.0), 20% (v/v) glycerol, 1 mM dithiothreitol, 4 mM MgCl₂, 1 µM pepstatin; buffer D, 20 mM potassium phosphate (pH 7.0), 30% (v/v) glycerol, 50 mM KCl, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 4 mM Na₂SO₄, 1 µM pepstatin; buffer E, 10 mM potassium phosphate (pH 7.0), 20% (v/v) glycerol, 1 mM dithiothreitol, 1 µM pepstatin, and 5 mM Na₂SO₄.

**Methods**

**Preparation of DNA Helicase Substrates**

The 17-mer universal sequencing primer (2.4 ng) was hybridized to ssM13 (mp9) DNA (1 µg) in 10 mM Tris-HCl (pH 7.8), 2.5 mM MgCl₂, 1 mM NaCl. Elongation of the primer was performed with 2 units of DNA polymerase I Klenow fragment by using 50 µM dGTP and dATP and 5 µCi of [α-32P]dCTP (3000 Ci/mmol). Unincorporated radioactivity was removed by gel filtration on Sephadex G-50 in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA. Thus the 17-mer primer was elongated by seven nucleotides resulting in a subsequence with a 24-mer double-stranded region. Similarly the directionality substrates were constructed by hybridizing the 30-mer Xhol-EcoRl polymer oligo to ssM13mp11. 3′ labeling was achieved by elongation with Klenow fragment resulting in a labeled 34-mer 5′ labeling was achieved with polynucleotide kinase and [γ-32P]ATP according to Maniatis et al. (30). Both substrates were cut with StuII resulting in the constructs shown in Fig. 9B.

**Enzyme Assays**

**DNA-dependent NT-Pase—DNA-dependent NT-Pase activity was determined as described earlier by using activated calf thymus DNA or ssM13 DNA (10 µg/ml) as DNA effector and either [2,5,6-3H] ATP or [α-32P]dCTP as nucleotides to be hydrolized (27).**

**DNA Helicase—DNA helicase activity assays were performed in a final volume of 25 µl containing: 20 mM Tris-HCl (pH 7.5), 4% (v/v) sucrose, 8 mM dithiothreitol, 80 µg/ml BSA, 1 mM MgCl₂, 1 mM ATP, and 2.0 ng of DNA substrate (3000 cpm/pmol). Incubation was for 60 min at 37 °C and was stopped by addition of 30 mM EDTA, 40% (v/v) glycerol, 0.25% (w/v) SDS, 0.05% (w/v) xylene cyanol, and 0.02% (w/v) bromphenol blue. The reaction products were separated by electrophoresis through a 12% native polyacrylamide gel in Tris-borate/EDTA buffer (pH 8.3) at 70 V. After electrophoresis the DNA was fixed in 10% (w/v) trichloroacetic acid and the gel dried and autoradiographed on Kodak X-omatic film. The autoradiogram served to identify the position of the substrate and the displaced fragment in the gel. After excision of the radioactive bands the amount of radioactivity in the position of the substrate and the displaced primer was quantified by liquid scintillation counting as described (32). 1 unit of helicase activity is defined as the amount of enzyme required to displace 50% of the radioactivity under the standard assay conditions.

**DNA Polymerase α—DNA polymerase α activity was determined in a final volume of 25 µl containing 20 mM potassium phosphate (pH 7.2), 0.1 mM EDTA, 4 mM dithiothreitol, 80 µg/ml BSA, 10 mM MgCl₂, 48 µM each of dATP, dCTP, and dGTP, 18 µM [γ-32P]dTPP (150 cpm/pmol), 3 µg of activated DNA, and enzyme to be tested. The amount of radioactivity that could be precipitated by trichloroacetic acid was determined as described (33).**

**Purification of DNA Helicase**

All operations were carried out at or near 0 °C unless otherwise noted.

**Crude Extract—1 kg of freshly harvested calf thymus was pulsed at ~20 °C by a Sorvall Omnimixer and resuspended in 5 liters of Buffer A for thawing. Crude extract was prepared exactly as described (34).**

**Phosphocellulose—**The crude extract was adsorbed onto a 1.2-liter phosphocellulose column (12 × 10.6 cm) previously equilibrated in buffer B. The column was first washed with 2.5 liters of buffer B and 5 liters of buffer B containing 100 mM potassium phosphate. Solution of DNA helicase (measured as DNA-dependent ATPase), DNA polymerase α-primase, and DNA polymerase ε was performed with 5 liters of buffer B containing 350 mM potassium phosphate. The peak fractions containing >60% of the three enzymatic activities were pooled and extensively dialyzed against buffer C containing 10 mM potassium phosphate.

**Hydroxyapatite—**The dialyzed phosphocellulose activities were adsorbed to a 150-ml (3.5 × 15 cm) hydroxyapatite column previously equilibrated in buffer C. After extensive washing a two-step elution was carried out. First, DNA polymerase α was eluted in buffer C containing 150 mM potassium phosphate. The active fractions of the second step (>60%) were pooled and dialyzed against buffer D.

**Heparin-Sepharose—**The hydroxyapatite fractions were adsorbed onto a 10-ml heparin-Sepharose column (2.7 × 6.4 cm) previously equilibrated in buffer D. The column was washed with two column volumes of buffer D containing 100 mM KCl. DNA helicase and DNA polymerase α-primase were eluted with a gradient of 10 column volumes ranging from 100 to 500 mM KCl in buffer D. On this column separation of DNA helicase from another DNA-dependent ATPase and DNA polymerase α was achieved (see “Results”). The fractions containing DNA helicase activity were pooled and dialyzed against buffer D.

**ATP-agarose—**The heparin-Sepharose fractions were loaded onto a 10-ml ATP-agarose column (1.6 × 5 cm) previously equilibrated in buffer D. The column was washed with three column volumes of buffer D containing 100 mM KCl. DNA helicase was eluted with a gradient of 10 column volumes containing 100–600 mM KCl in buffer D. The active fractions were pooled and dialyzed against buffer E.

**FPLC-Mono S—**The pooled and dialyzed fractions from the ATP-agarose column were applied to an 1-ml FPLC-Mono S column equilibrated in buffer E. The fractions containing DNA helicase activity were pooled and dialyzed against buffer D.

**Velocity Gradient Centrifugation**

DNA helicase peak fractions of the Mono S column (100 units) were dialyzed against 30 mM potassium phosphate (pH 7.0), 50 mM KCl, 1 mM dithiothreitol, 1 µM pepstatin, 5 mM Na₂SO₄, and layered onto a preformed 5-ml 10-30% (v/v) glycerol gradient prepared in the same buffer. Centrifugation was at 44,000 rpm for 28 h in a TST-54 rotor (Sorvall) at 4 °C. Fractions of 200 µl were collected from the bottom of the tubes and assayed for DNA helicase activity. The fractions containing the peak activity were individually stored in
small aliquots in liquid nitrogen. Under these storage conditions DNA helicase activity remained stable for at least 4 months.

**Gel Filtration in Superose**

Superose 6 (prep grade) was packed into a 1 × 30-cm column according to the manufacturer’s protocol (Pharmacia LKB Biotechnology Inc.). The column was equilibrated in 50 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 50 mM NaCl, 2.5 mM dithiothreitol, 0.5 mM EDTA, and 1 μM pepstatin. The peak fractions of the Mono S column were concentrated three times against the same buffer by pressure dialysis (Schleicher & Schuell). 500 μl (1000 units) were loaded onto the column and filtrated at a speed of 0.05 ml/min. Fractions of 200 μl were collected and assayed for DNA helicase activity.

**Other Methods**

Protein determination and SDS-polyacrylamide gel electrophoresis were performed according to Bradford (35) and Laemmli (36), respectively. Silver staining was performed as outlined in Ref. 37. The sedimentation coefficient and the Stokes radius were calculated according to Ref. 38.

**RESULTS**

Comments on Detection and Purification of Calf Thymus DNA Helicase—Upon conventional purification of a DNA polymerase α-primase (34,39) or an even more complex DNA polymerase δ holoenzyme (40) we realized that a DNA-dependent ATPase copurified with DNA polymerase α-primase but never with DNA polymerase ε (34,39). This can be demonstrated best during the simultaneous isolation of DNA polymerases α and δ where DNA-dependent ATPase activities copurified on phosphocellulose with DNA polymerases α and ε and on hydroxylapatite with DNA polymerase α-primase only, while DNA polymerase ε was separated from the latter (Fig. 1 and Ref. 34). In a completely different purification procedure, which allows the simultaneous purification of DNA polymerases α, δ, and ε, the helicase purified together with DNA polymerase α on phenyl-Sepharose column, while it was separated from DNA polymerases δ and ε. Final separation of the DNA-dependent ATPases from DNA polymerase α-primase was achieved on heparin-Sepharose (Fig. 2). However, when these ATPase peak fractions, which were completely dependent on DNA for ATP hydrolysis (data not shown), were tested in a strand displacement assay, little DNA helicase activity was found (Fig. 2). While at this stage of purification the pool containing the DNA-dependent ATPase still was contaminated by nucleases (Fig. 2) it showed no helicase activity even after further purification and separation from the nucleases (data not shown). However, the fractions between the main DNA-dependent ATPase and DNA polymerase α-primase peak had significant DNA helicase activity (Fig. 2). These fractions were pooled and purified to apparent homogeneity by chromatography through ATP-agarose and FPLC-Mono S (Table 1). The enzyme was purified from hydroxylapatite to FPLC-Mono S to a specific activity of 15,500 units/mg with a yield of 9%. At the early stages of purification (up to hydroxylapatite) it was not possible to determine DNA helicase activity exactly due to the contamination with nucleases (see Fig. 2). On the ATP-agarose column a persistent unspecific nuclease activity could finally be

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2 A revised nomenclature for eukaryotic DNA polymerases has been proposed recently (49). (i) The greek letter nomenclature is adopted for yeast DNA polymerases as well as for the mammalian enzymes; (ii) the DNA polymerase δ dependent on proliferating cell nuclear antigen is called DNA polymerase δ (formerly DNA polymerase III in yeast); and (iii) the proliferating cell nuclear antigen-independent DNA polymerase δ is called DNA polymerase ε (formerly DNA polymerase II in yeast).


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**Fig. 1.** Flow diagram for simultaneous isolation of DNA helicase, DNA polymerase ε, proliferating cell nuclear antigen, and DNA polymerase α-primase.

**Fig. 2.** Separation of calf thymus DNA helicase from DNA-dependent ATPase and DNA polymerase α-primase by heparin-Sepharose. DNA helicase was purified in the heparin-Sepharose step as described under “Experimental Procedures.” A, fractions of the column were assayed for DNA helicase (●), DNA-dependent ATPase (×), and DNA polymerase α (○). B, autoradiogram of the helicase assay using 1 μl each of fractions 47, 64, and 80.
**Table I**

Purification of calf thymus DNA helicase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>DNA helicase activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>Total units</td>
<td>Specific units/mg</td>
</tr>
<tr>
<td>Crude extract</td>
<td>30,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>1,085</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>482</td>
<td>192,000</td>
<td>398</td>
</tr>
<tr>
<td>Heparin-Sepharose</td>
<td>35</td>
<td>54,000</td>
<td>1,500</td>
</tr>
<tr>
<td>ATP-agarose</td>
<td>17</td>
<td>24,800</td>
<td>1,500</td>
</tr>
<tr>
<td>FPLC-Mono S</td>
<td>1.14</td>
<td>17,480</td>
<td>15,500</td>
</tr>
</tbody>
</table>

*DNA helicase activity cannot be determined at these stages because the fractions contain too much nuclease activity.*

**Fig. 3.** Removal of contaminating nuclease from calf thymus DNA helicase by ATP-agarose column. DNA helicase was purified to the ATP-agarose step as described and tested for DNA helicase activity. The amount of primer that remained hybridized to ssM13 (C, remaining) or was displaced by helicase action (O, displaced) was quantified as described under "Experimental Procedures." If the sum of displaced and remaining radioactivity (x) is significantly less than 100% of the control values (without enzyme and after denaturation at 100°C), it is indicative for a nuclease contamination (compare fractions 20-30).

**Biochemical and Physical Properties of Calf Thymus DNA Helicase**—The purified DNA helicase preparation showed a 47-kDa polypeptide following the peak of activity after velocity sedimentation in a glycerol gradient (Fig. 4) or after gel filtration in Superose 6. Based upon silver staining of the gradient fractions we estimate the purity of the enzyme over 99%. The DNA helicase had a broad sedimentation coefficient of 4-7 S. This value remained the same if the enzyme was sedimented in a high salt gradient (1 M KCl; data not shown; see "Discussion"). Gel filtration in Superose 6 resulted in an enzymatic activity eluting between BSA and ovalbumin with a Stokes radius of about 45 Å (Fig. 5).

**Reaction Requirements of the Calf Thymus DNA Helicase**—Table II shows that the DNA helicase requires MgCl₂ for activity with a broad optimum concentration between 0.5 and 5 mM. In contrast, MnCl₂ was effective to 60% compared to MgCl₂, but only in a concentration range between 1 and 2 mM. CaCl₂ was never more effective than 20% in the whole concentration range used (up to 10 mM). KCl and NaCl at concentrations of 200 mM inhibited 75% of the activity. The reaction required the hydrolysis of the γ-phosphate of ATP, since ATP-γ-S and the nonhydrolyzable analog AMP-PCP
DNA Helicase from Calf Thymus

FIG. 5. Gel filtration on FPLC-Superose 6. DNA helicase (1000 units) of FPLC-Mono S fraction was concentrated by pressure dialysis and filtered on a 23-ml Superose 6 column (1 x 30 cm) at a flow rate of 3 ml/h. Fractions were analyzed for DNA helicase and dCTPase activity as described under "Experimental Procedures." A, quantification of helicase activity on the column in the presence of ATP (●) and hydrolysis of dCTP (○). Markers were: 1, dextran blue; 2, thyroglobulin (669 kDa); 3, ferritin (440 kDa); 4, catalase (232 kDa); 5, aldolase (158 kDa); 6, BSA (67 kDa); and 7, ovalbumin (43 kDa). B, autoradiograms of helicase assays on the column in the presence of either ATP, ddATP, CTP, and ATP+ (1 mM each).

TABLE II

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>+ Helicase</td>
<td>&lt;1</td>
</tr>
<tr>
<td>+ Helicase (heated 10 min, 65 °C)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>+ MgCl₂</td>
<td>2</td>
</tr>
<tr>
<td>+ EDTA (1 mM)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>+ MnCl₂ (1 mM), -MgCl₂</td>
<td>59</td>
</tr>
<tr>
<td>+ CaCl₂ (1 mM), -MgCl₂</td>
<td>13</td>
</tr>
<tr>
<td>+ NaCl (200 mM)</td>
<td>25</td>
</tr>
<tr>
<td>+ KCl (200 mM)</td>
<td>26</td>
</tr>
<tr>
<td>+ ATP</td>
<td>3</td>
</tr>
<tr>
<td>+ AMP-PCP</td>
<td>&lt;1</td>
</tr>
<tr>
<td>+ ATPγS</td>
<td>&lt;1</td>
</tr>
<tr>
<td>+ Gene 32 protein (75 ng)</td>
<td>98</td>
</tr>
<tr>
<td>+ Histone H1 (25 ng)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>+ Nucleolin (calf thymus, 150 ng)</td>
<td>86</td>
</tr>
</tbody>
</table>

were inactive as effectors. Compared to ATP other nucleoside 5'-triphosphates could be utilized by the calf thymus DNA helicase to a lesser extent (Table III); dATP supported more than 80% of the activity (see also Fig. 6), while CTP, dCTP, and dideoxy-ATP could partially serve as effectors. GTP, dGTP, UTP, and dTTP all were inactive. The $K_m$ for ATP and dATP were 0.2 and 0.25 mM, respectively. Fig. 6 documents the titration of ATP and dATP and compares it to the titration of ATPγS and AMP-PCP. Again the nonhydrolyzable analogs were completely ineffective at all concentrations tested (0.2-2 mM). From Fig. 5 it is furthermore evident that strand displacement activity of calf thymus DNA helicase comigrates with a DNA-dependent dCTPase upon filtration.

TABLE III

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Activity %</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100</td>
<td>0.20</td>
</tr>
<tr>
<td>CTP</td>
<td>63</td>
<td>0.45</td>
</tr>
<tr>
<td>GTP</td>
<td>&lt;1</td>
<td>ND*</td>
</tr>
<tr>
<td>UTP</td>
<td>&lt;1</td>
<td>ND</td>
</tr>
<tr>
<td>dATP</td>
<td>82</td>
<td>0.25</td>
</tr>
<tr>
<td>dCTP</td>
<td>20</td>
<td>ND</td>
</tr>
<tr>
<td>dGTP</td>
<td>&lt;1</td>
<td>ND</td>
</tr>
<tr>
<td>dTTP</td>
<td>&lt;1</td>
<td>ND</td>
</tr>
<tr>
<td>ddATP</td>
<td>40</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND, not determined.
through a Superose column. On the same column DNA helicase activity dependent on CTP and ddATP is present in the same fractions as the ATP-dependent helicase activity, while in the presence of ATPγS, no displacement was evident.

**Characterization of the Calf Thymus DNA Helicase**—Titration of DNA helicase under optimal assay conditions, using 2.5 ng of substrate, shows saturation at 90 ng of protein (Fig. 7). With 200 ng of enzyme the displacement reached a level of more than 90% (Fig. 7A). If an amount that gives 60% displacement was used for a time course (Fig. 7B) the displacement of this 24-base fragment was linear for 20 min and saturated at 50 min. Under these conditions no measurable reassociation of the substrate took place (data not shown). The DNA helicase reaction can be competed with nonradioactive ssM13 DNA and primed ssM13 DNA (Fig. 8). 50% inhibition was achieved when the competitor:substrate ratio was 1:1. Double-stranded pBR322 on the other hand could not compete with the substrate in the displacement reaction.

DNA restriction fragments with either blunt or sticky ends also could not compete (data not shown). This experiment suggests that calf thymus DNA helicase can bind to single-stranded but not to double-stranded DNA. A certain length of ssDNA appears to be required for binding. Finally, the two ssDNA binding proteins, the gene 32 protein from bacteriophage T4, and nucleolin from calf thymus (41) had no effect on the DNA helicase activity. On the other hand calf thymus histone H1 completely inhibited the helicase activity (Table II).

**Directionality of Unwinding**—To determine the direction of unwinding the two substrates shown in Fig. 9B were constructed. The 30-base Xbal-EcoRI polylinker oligo was labeled at the 5′ end by polynucleotide kinase and [γ-32P]ATP and hybridized to ssM13mp11 DNA. Alternatively the primer was hybridized to ssM13mp11 DNA and elongated at the 3′ end by DNA polymerase I Klenow fragment in the presence of [α-32P]dCTP but in the absence of dATP resulting in a labeled 34-mer fragment. Both products were cut with SmaI to create a substrate with either a 5′-labeled 16-base fragment or a 3′-labeled 18-base fragment. Unwinding by calf thymus DNA helicase is in the 3′→5′ direction since it exclusively displaces the labeled 18-base fragment (Fig. 9A). As expected, SV40 T-antigen displaces the same fragment indicating the same 3′-, 5′ directionality (24). In contrast DNA helicase I from E. coli, an enzyme known to unwind in the 5′→3′ directionality (17), displaces the 18-base fragment. No displacement of this fragment was seen with either calf thymus DNA helicase or with SV40 T-antigen.

**Effect of DNA Polymerase α-Primase on the Strand Displacement of DNA Helicase**—Since we realized that a substantial loss of DNA helicase activity was seen when DNA polymerase α-primase was separated from the DNA helicase (Table I), we tested a relative crude DNA helicase fraction still containing DNA polymerase α-primase (heparin-Sepha-
rose fraction 80 of Fig. 2) in the presence of inhibitors and antibodies of DNA polymerase α. No inhibitory effect was seen in the presence of aphidicolin, BuPdGTP, and the DNA polymerase-specific neutralizing monoclonal antibody SJK152-20 (Fig. 10). Addition of DNA polymerase α-primase to subsaturating amounts of DNA helicase (FPLC-Mono S fraction, Table I) gave no stimulation (data not shown). Taken together these data suggest that DNA polymerases
Fig. 9. Directionality of unwinding by calf thymus DNA helicase. A, DNA helicase assays were carried out as described under "Experimental Procedures" by using 2.5 ng of substrate DNA, 80 ng of calf thymus DNA helicase, 2.2 μg of DNA helicase I from E. coli, and 300 ng of SV40 T-antigen. B, DNA substrates were prepared as outlined under "Experimental Procedures."  

FIG. 10. Effect of DNA polymerase α inhibitors on calf thymus DNA helicase activity. Heparin-Sepharose fraction 80 containing DNA polymerase α and helicase activity (see Fig. 2 for details) was used in a strand displacement assay as described under "Experimental Procedures." The inhibitor indicated at the top of each lane was added to the reaction: BuPdGTP (5 μM), monoclonal antibody SJK132-20 (2 μg), aphidicolin (100 μg/ml).  

DISCUSSION  

A DNA helicase from calf thymus was purified to apparent homogeneity. The results suggest that the purified enzyme has a mass of 47 kDa and might act as a monomer (Figs. 4 and 5). The broad sedimentation peak in the glycerol gradient (Fig. 4) which is salt-independent hints at the possibility that two populations of the enzyme might exist, a monomeric and an aggregated one. The DNA helicase has a relatively low Kₘ for ATP (Table III) and requires hydrolysis of ATP presumably used for the displacement of DNA (Table II and Figs. 5 and 6). The enzyme appears to bind to single-stranded DNA (Fig. 8), to move in a 3'→5' direction on the DNA it is bound to, and to unwind in the same direction (Fig. 9).  

The only DNA helicases that are so far homogeneous and well characterized are the large T-antigen from SV40 (23, 24) and the DNA helicase from herpes simplex (25, 26). However, both are gene products of viral origins. Comparison of the described calf thymus DNA helicase to other eukaryotic DNA helicases is difficult since the DNA helicases from cellular origin of vertebrates (calf thymus, mouse cells, and Xenopus laevis oocytes) were not homogenous enough nor characterized in detail (27–29). The ATPase B from mouse FM3A cells (28) is a DNA helicase that has been separated from several DNA-dependent ATPases, partially purified, and characterized as a DNA helicase. The DNA helicase described earlier from our laboratory (27) had a similar behavior as the one characterized here by its tendency to copurify with the DNA polymerase α-primase complex during at least some of the purification steps. Similarly, an association of the T-antigen helicase with DNA polymerase α has been described (42); also, the direction of movement of T-antigen is the same as for the calf thymus DNA helicase. On the other hand, strand displacement activity also could be separated from calf thymus DNA polymerase δ and identified as DNA helicase (43).  

What about the putative in vivo function of this enzyme? Due to the lack of a conditional lethal mutant there is an uncertainty in making a definitive prediction of the function of this enzyme. However, biochemical characteristics and the direction of unwinding might suggest a possible role at the replication fork. First, the enzyme has a low Kₘ for hydrolysis of nucleoside 5'-triphosphate (200 μM for ATP and 250 μM for dATP) compared to other DNA helicases, such as SV40 T-antigen helicase with a Kₘ of 600 μM for ATP (24). We do not know, however, the concentration of deoxyribonucleosides and nucleoside 5'-triphosphates at the replication fork. It might well be that the concentration of 200 μM ATP, which is the universal energy donor in the cell, could be high enough in the nucleus to provide the DNA helicase with ATP. In addition calf thymus DNA helicase is also able to use dCTP and CTP as nucleoside 5'-triphosphates (Table III). Mammalian cells can be labeled in the G₀ phase with [5-3H] deoxycytidine to high specific activities (44) indicating a dCTP pool which is much higher than the one of the other deoxyribonucleoside triphosphates. Whether this potentially high dCTP concentration might eventually be used by the DNA helicase is an attractive hypothesis but purely speculative at this time. Second, the 3'→5' directionality of unwinding could suggest a possible role for the calf thymus DNA helicase during the early events of DNA unwinding similarly as it has been described for SV40 T-antigen. This hints at the possibility that the mammalian DNA helicase might be part of the primosomal complex as proposed recently for E. coli dnaB protein (11) and the T4 gene 41 proteins (45), as well as for the DNA helicase from herpes simplex virus (26). Construction of a preformed replication fork (46) on which DNA polymerase α-primase (40), DNA polymerase δ (47), proliferating cell nuclear antigen, and the DNA helicase described here can act will give more information on the possible functions of these enzymes in eukaryotic DNA replication (48).  

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REFERENCES  

DNA Helicase from Calf Thymus


