A DNA helicase from human cells

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ABSTRACT

We have initiated the characterization of the DNA helicases from HeLa cells, and we have observed at least 4 molecular species as judged by their different fractionation properties. One of these only, DNA helicase I, has been purified to homogeneity and characterized. Helicase activity was measured by assaying the unwinding of a radioactively labelled oligodeoxynucleotide (17 mer) annealed to M13 DNA. The apparent molecular weight of helicase I on SDS polyacrylamide gel electrophoresis is 65 KDa. Helicase I reaction requires a divalent cation for activity (Mg^{2+} > Mn^{2+} > Ca^{2+}) and is dependent on hydrolysis of ATP or dATP. CTP, GTP, UTP, dCTP, dGTP, dTTP, ADP, AMP and non-hydrolyzable ATP analogues such as ATPγS are unable to sustain helicase activity. The helicase activity has an optimal pH range between pH8.0 to pH9.0, is stimulated by KCl or NaCl up to 200mM, is inhibited by potassium phosphate (100mM) and by EDTA (5mM), and is abolished by trypsin. The unwinding is also inhibited competitively by the coaddition of single stranded DNA. The purified fraction was free of DNA topoisomerase, DNA ligase and nuclease activities. The direction of unwinding reaction is 3′ to 5′ with respect to the strand of DNA on which the enzyme is bound. The enzyme also catalyses the ATP-dependent unwinding of a DNA:RNA hybrid consisting of a radioactively labelled single stranded oligodeoxynucleotide (18 mer) annealed on a longer RNA strand. The enzyme does not require a single stranded DNA tail on the displaced strand at the border of duplex regions; i.e. a replication fork-like structure is not required to perform DNA unwinding. The purification of the other helicases is in progress.

INTRODUCTION

DNA helicases catalyze the unwinding of double stranded DNA to provide single-stranded templates for DNA replication, repair and recombination utilizing the energy provided by the hydrolysis of the γ-phosphate of ATP (1). Up to now many proteins with DNA helicase activity have been isolated and characterized from various sources such as bacteriophage infected cells (1, 2, 3), *Escherichia coli* (4–7), mammalian virus infected cells (8, 9), yeast (10, 11), lily (12), xenopus laevis (13), calf thymus (14, 15), mouse (16) and human cells (17, 18, 19). As for their physiological role, among the eight described DNA helicases of *E. coli*, helicase I is required for conjugal DNA transfer (20), helicase II is involved in excision repair (21) and methyl directed mismatch repair (22), the n′ protein (6) and *dna*B protein (23) are involved in DNA replication, and the *rec*BCD enzyme in recombination (24). Some DNA helicases stimulate DNA synthesis by DNA polymerase (10, 17, 18, 25).

In addition to DNA helicases, RNA helicases have also been described; the *E. coli* transcription termination factor rho has been shown to possess RNA-DNA helicase activity (26) which presumably facilitates the release of RNA transcripts from the DNA template at rho dependant terminators. Also DNA helicase II of *E. coli* is capable of disrupting an RNA-DNA helix (27). In eukaryots RNA-DNA helicase activity has been shown in mammalian translation initiation factor e1F-4A (28), human p68 protein (29), and SV40 T antigen (30). Recently both RNA-RNA and RNA-DNA helicases have been shown to contain sequence similarities with several prokaryotic DNA helicases (31, 32).

All the helicases studied for their mechanism of action have been shown to translocate unidirectionally along the single-stranded (ss) DNA to which they are bound (7, 9). Most of the helicases need ss DNA adjacent to the duplex region to be unwound,with the notable exception of the SV40 TAg (30).

Less is known about helicases present in eukaryotic cells and in particular about human DNA helicases. We had previously observed (17, 18) that DNA-dependant ATPase preparations from human cells also contained DNA unwinding activity, at least in partially purified fractions; we have now initiated a systematic study of human DNA helicases. In this work we show that HeLa cells contain several forms of DNA helicases, one of which, named human DNA helicase I (HDH I), we have purified to homogeneity. We report here the purification procedure and the main properties of HDH I.

MATERIALS AND METHODS

Reagents

All reagents were of enzyme grade. Phenylmesulfonyl fluoride (PMSF), sodium bisulfite, bovine serum albumin, DTT, HEPES and EDTA were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Ultrapure grade Tris, ammonium sulfate, single-stranded (+ strand) and RFI DNA of M13mp19, fetal calf serum, donor calf serum, glutamine and gentamycin were from Gibco-BRL Corp. (Gaithersburg, MD, USA). All electrophoresis reagents, protein markers, silver stain kit and Bio Rex 70 resin

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were from Bio-Rad Corp. (Richmond, CA, USA). DEAE-Sephacel, Sepharose 4B, Heparin sepharose, CNBr activated sepharose 4B, all the chromatographic columns, nonradioactive nucleoside triphosphates, ADP and AMP were from Pharmacia Corp. (Uppsala, Sweden). [γ^{32}P]ATP (5000 Curie/mmol) and [α^{32}P]dCTP (3000 Curie/mmol) were from Amersham (Amersham, UK). Restriction endonucleases, DNA polymerase I (large fragment) and bacteriophage T4 polynucleotide kinase were from New England Biolabs Corp. (New England, USA). T7 RNA polymerase, RNase-free DNase and RNase block were from Stratagene Corp. (La Jolla, CA, USA). Adenosine 5'-0-(3-thiotriphosphate) (ATPγS), was from Boehringer Mannheim GmbH (Mannheim, FRG). Trypsin was from Serva (Heidelberg, FRG). The oligodeoxynucleotides used for making DNA helicase substrates, reported in Table 1, were synthesised using an Applied Biosystems 380 A DNA synthesizer.

Preparation of 5'-labelled helicase substrate

The DNA substrate used in helicase assays consists of ^{32}P labelled complementary oligodeoxynucleotides annealed to single-stranded M13mp19 phage DNA to create a partial duplex. Oligodeoxynucleotide 1 (17-mer), table 1, was mainly used except as otherwise stated. A total of 100 ng of oligodeoxynucleotide was labelled at the 5' -end with T4 polynucleotide kinase and [γ^{32}P]ATP (33) and ethanol precipitated twice to remove free nucleotides. The labelled oligodeoxynucleotide was then annealed with 2.5μg of single-stranded circular M13mp19 DNA in 20mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 100mM NaCl and 1mM DTT by heating at 95°C for 1 min, transferring immediately to 65°C for 2 min and then cooling slowly to room temperature. Non-hybridized oligodeoxynucleotide was removed by gel filtration through a 1 ml sepharose 4B column with 10mM Tris-HCl (pH 7.5), 1mM EDTA and 100mM NaCl.

Preparation of 3'-labelled helicase substrate

Oligodeoxynucleotide 5 (41-mer, 100ng), table 1,was annealed to 2.5μg of single stranded circular M13mp19 DNA in 40 mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 50mM NaCl and 1mM DTT. The mixture was first heated at 95°C for 1 min and then allowed to anneal at 65°C for 20 min. The resulting partial duplex was labelled at the 3'-OH end in 40mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 50mM NaCl and 1mM DTT with 50μCurie [α^{32}P]dCTP and 5 units of DNA polymerase I (large fragment) at 23°C for 20 min. The dCTP was subsequently increased to 50mM using unlabelled dCTP and the incubation was continued for an additional 20 min at 23°C. Unreacted radionucleotides were removed by gel filtration through 1 ml of Sepharose 4B.

DNA helicase assay

The helicase assay measures the unwinding of α ^{32}P labelled DNA fragment from a partial duplex DNA molecule (Fig. 1). The reaction (10μl) containing 20mM Tris-HCl (pH 9.0), 8mM DTT, 2mM $MgCl_2$, 2mM ATP, 10mM KCl, 4% (wt/vol) sucrose, 80 mg/ml BSA, ^{32}P-labelled helicase substrate (1000 cpm) and the helicase fraction to be assayed was incubated at 37°C for 20 min (unless otherwise indicated) and terminated with 0.3% SDS, 10mM EDTA, 5% glycerol and 0.1 mg/ml bromophenol blue. After further incubation at 37°C for 5 min the substrate and product were separated by electrophoresis on a 12% nondenaturing polyacrylamide gel. The gel was dried under vacuum and exposed to Amersham Hyperfilm MP with an intensifying screen for autoradiography. DNA unwinding was quantitated by excising the bands from the dried gel and counting in Beckman Ready Safe liquid scintillation fluid. One unit of DNA helicase activity is defined as the amount of enzyme unwinding 1% of the DNA helicase substrate in 1 min at 37°C (20% in the 20 min reaction).

Preparation of blunt ended duplex DNA helicase substrate

Oligodeoxynucleotide 5 (41-mer, 100ng), table 1,was 5'-end labelled with T4 polynucleotide kinase and [γ^{32}P]ATP as described above. This labelled 41-mer was annealed with oligodeoxynucleotide 6 (100ng), table 1, in 40mM Tris-HCl (pH 7.5), 20mM $MgCl_2$ and 50mM NaCl. The annealing mixture

Table 1. Oligodeoxynucleotides synthesized for the preparation of helicase substrates.

Oligodeoxynucleotides	Sequence	Note
1 (17-mer)	5'-GTAAAACGACGGCCAGT-3'	complementary to M13mp19 DNA;
2 (60-mer)	5'-$(T)_{10}$CGAGCTCGGTACCCGGGGATCC TCTAGAGTCGACCTGCA$(T)_{11}$-3'	The middle 39 nucleotides are complementary to M13mp19 DNA;
3 (45-mer)	5'-GTTCCAGCGCTAGCTTCGAGCTCGGT ACCCGGGGATCCTCTAGAG-3'	the last 31 nucleotides are complementary to M13mp19 DNA ;
4 (44-mer)	5'- TCGAGCTCGGTACCCGGGGATCCTCT AGAG$(T)_{14}$-3'	the first 31 nucleotides are complementary to M13mp19 DNA;
5 (41-mer)	5'- AATTCGAGCTCGGTACCCGGGGATCC TCTAGAGTCGACCTG-3'	complementary to M13mp19 DNA;
6 (41-mer)	5'-CAGGTCGACTCTAGAGGATCCCCGGGT ACCGAGCTCGAATT-3'	complementary to oligodeoxynucleotide 5 (41-mer) and also complementary to the middle part of oligodeoxynucleotide 2 (60-mer);
7 (42-mer)	5'-TCGAGCTCGGTACCCGGGGATCCTCTA GAGTCGACCTGCAGG-3'	complementary to M13mp19 DNA;
8 (18-mer)	5'- GATGCCATATTGGGCCAG-3'	complementary to a 1 kb cDNA (position 241−258) of the γ subunit of human retinal cGMP phosphodiesterase (34), used for making the RNA-DNA substrate.

was heated to 65°C for 2 min and allowed to cool slowly to room temperature. Finally this mixture was passed through 1 ml Sepharose 4B column to remove unreacted radionucleotides.

Preparation of substrate for determination of helicase directionality

Oligodeoxynucleotide 7 (42-mer), table 1, was labelled at the 5'-end by [γ^{32}P]ATP and annealed with single-stranded circular M13mp19 DNA as described above in preparation of 5'-end labelled substrate. The annealed DNA was labelled at the 3'-end by [α^{32}P] dCTP as described above in preparation of 3'-end labelled substrate, digested with SmaI and passed through a 1 ml Sepharose 4B column to remove unreacted radionucleotides (see below in the Results section).

Preparation of RNA-DNA substrate

The plasmid bluescript (KS+) containing γ-subunit of human retinal cGMP phosphodiesterase cDNA (34) inserted in the EcoR1 site has been used for RNA transcription. This plasmid was linearized with SmaI and then used for synthesis of nonradioactive RNA using T7 RNA polymerase as described in the Stratagene instruction manual. After the transcription reaction, the RNA-DNA hybrid was treated with RNase-free DNase, extracted with phenol:chloroform (1:1) and precipitated with ethanol to produce an RNA strand for use in preparing an RNA-DNA substrate (see below in the Results section). Oligodeoxynucleotide 8 (18-mer, 50ng), table 1, was labelled at the 5'-end with [γ^{32}P]ATP as described above and annealed with 1μg of RNA (*in vitro* transcribed) in 20μl containing 25mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 50mM NaCl and 20 units of RNase block at 65°C for one hour. The unreacted radionucleotides were removed by gel filtration through 1ml of Sepharose 4B.

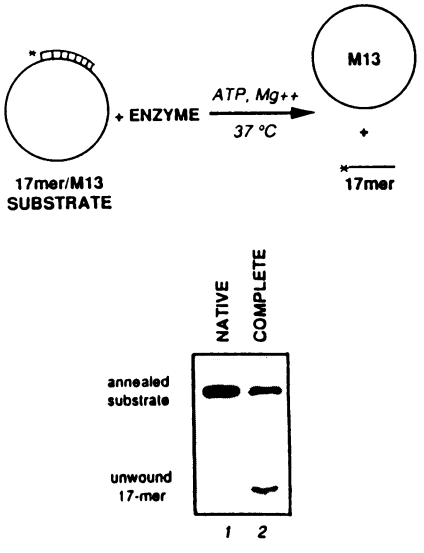

Figure 1. Scheme for measuring helicase activity. Preparation of the substrate and helicase assay condition are described under 'Materials and Methods''. Top, the ^{32}P labelled 17-mer is hybridized to ssM13 mp19 DNA. This substrate is incubated with the sample containing helicase, which unwinds the 17-mer fragment from the ssDNA circle. Bottom, the products are separated by electrophoresis in a 12% nondenaturing polyacrylamide gel. The annealed substrate does not enter the gel, the unwound fragment migrates in the gel; autoradiography reveals the position of the labelled fragment. Lane 1: reaction without the enzyme. Lane 2: reaction with, enzyme (5 ng).

Buffers

All buffers used during purification contained 1mM PMSF and 1mM sodium bisulfite. Buffer A contained 20mM HEPES (pH 8.0), 0.1M NaCl, 1mM DTT, 1mM EDTA and 20% glycerol. Buffer B contained 50mM Tris-HCl(pH 8.0), 50mM KCl, 1mM DTT, 1mM EDTA and 10% glycerol. Buffer C was buffer B except with 0.1M KCl. Buffer D was buffer B with 0.15M KCl and 1mM $MgCl_2$.

Cell cultures

HeLa cells were grown in Joklin MEM supplemented with 5% fetal calf serum, 5% donor calf serum, 2mM glutamine and 50μg/ml gentamycin. When the cell population, started at 2×10^5 cells/ml reached about 5×10^5 cells/ml (4 days), the cells were harvested by centrifugation, washed twice with Dulbecco's complete phosphate buffered saline, quickly frozen as a pellet in a dry ice-ethanol bath and stored at −80°C. One gram of the frozen pellet contained about 3×10^8 cells.

Other Methods

DNA dependent ATPase was assayed as described by Hubscher and Stadler (14). DNA topoisomerase assays were performed as described by Kaiserman et al. (35) except that the plasmid DNA used was Bluescript containing a cDNA (1 kb) of human cGMP phosphodiesterase (34). DNA nicking activity was assayed as described by Hughes et al. (36). DNA ligase activity was checked by ligation of EcoRI cut Bluescript and the cDNA insert of human retinal cGMP phosphodiesterase, followed by transformation (33). Protein determination and SDS polyacrylamide gel electrophoresis were performed according to Bradford (37) and Laemmli (38) respectively. Silver staining was done using Bio-Rad's silver staining kit.

RESULTS

Purification of human DNA helicase I (HDH I)

All purification steps were carried out at 0−4°C. The frozen cells (100g) were thawed on ice and a nuclear extract was prepared as described by Dignam et al. (39). The nuclear extract proteins were precipitated by slowly adding ammonium sulfate (0.35g/ml) with constant stirring. The mixture was stirred for an additional hour and the precipitate was collected by centrifugation.The pellet was resuspended and dialyzed in buffer A (fraction I: 49 ml). The supernatant also showed helicase activity (HDH IV: see below). The exact amount of activity present in fractions I and II could not be determined due to the presence of interfering nucleases.

Fraction I was passed through a Bio-Rex 70 column (2.5×33 cm) equilibrated with buffer A. The column was washed with 3 column volumes of buffer A and the non absorbed protein fractions having helicase activity (HDH I) were pooled (fraction II: 142 ml). The absorbed proteins from the column were eluted with a 5 column volume linear gradient of 0.1M to 0.6M NaCl in buffer A followed by step elution with 1M NaCl in buffer A. Protein fractions containing helicase activity eluted at about 0.4M and 1.0M NaCl and were named HDH II and HDH III respectively (Fig. 2). Here we describe the purification of HDH I; the purification of other helicases is in progress.

Fraction II (HDH I) was precipitated with ammonium sulfate (0.35g/ml) as described above. The protein pellet was resuspended and dialyzed in buffer B (fraction III: 8 ml; 53,124 units). Fraction III was loaded onto a DEAE-sephacel column

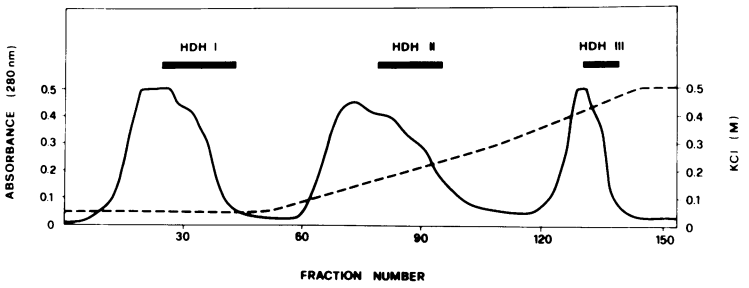

Figure 2. Elution profile of proteins on Bio-Rex 70. Detail description of this column is given in the text. Solid bars correspond to the pooled fractions containing DNA helicase activity. Only human DNA helicase I (HDH I) was further purified and characterized.

(1.6×14 cm) equilibrated with buffer B. The column was washed with 3 column volumes of buffer B and eluted with a 5 column volume linear gradient from 0.05M to 0.65M KCl in buffer B. The activity eluted at about 0.25M KCl. The active fractions were pooled (fraction IV; 20 ml, 41,340 units).

Fraction IV was diluted to 0.1M KCl with buffer B (without KCl) and loaded onto a heparin sepharose column (1.4×4 cm) equilibrated with buffer C. The column was washed with 3 column volumes of buffer C and subsequently eluted with 5 column volume linear gradient from 0.1M KCl to 1M KCl in buffer C. The activity eluted from the column at about 0.65M KCl. The active fractions were pooled and diluted to 0.15M KCl with buffer B having no KCl (fraction V; 11 ml, 35,250 units). To this fraction $MgCl_2$ was added up to a final concentration of 1mM.

Fraction V was loaded onto a single stranded (ss) DNA sepharose 4B column (1.6×2cm) equilibrated with buffer D. The ss DNA sepharose 4B was prepared as follows: genomic DNA was isolated from HeLa cells (33), sonicated by 15 pulses of 30 sec at 60 watt to an optimum size of 0.500−3.0 Kb as checked by agarose gel electrophoresis. This sonicated DNA was denatured in 0.2M NaOH and ethanol precipitated. This single stranded DNA (1 mg) was then coupled to CNBr activated sepharose 4B (1 ml packed) as described in the Pharmacia booklet. After loading fraction V, the column was washed with 3 column volumes of buffer D and subsequently eluted with a 6 column volume gradient of 0.15M to 1M KCl in buffer D. The activity eluted at about 0.9M KCl (fraction VI; 45 ml, 15,000 units). At this stage the HDH I was not stable at 4°C but could be stored in 25% glycerol at −80°C up to four months with little loss of activity.

Table 2 reports a summary of the purification. The HDH I activity which eluted at 0.9M KCl showed only one band on SDS polyacrylamide gel electrophoresis of about 65 kDa (Fig. 3, lane 2). Although some activity also eluted from the ss DNA sepharose column at 0.7 to 0.8M KCl, these fractions were not pure. Further attempts to purify these impure fractions resulted in a rapid loss of activity. Instability is probably the reason for the low recovery of total units of HDH I from the ssDNA sepharose column (Table 2). From 100g of HeLa cells we obtained 0.11 mg of pure HDH I enzyme with the specific activity of 136,362 units/mg. HDH I did not contain any detectable levels of nuclease, topoisomerase, DNA ligase or DNA nicking activities. DNA dependant ATPase activity was present at a level of approximately 0.014 pmols of ATP hydrolized in 20 min. by 5 ng of protein.

Characterization of HDH I

The helicase activity of HDH I was characterized by assaying the unwinding of radioactively labelled oligodeoxynucleotide annealed to circular M13 ss DNA, as described in the previous section (Fig. 1). The optimum temperature for the helicase reaction was found to be 37°C. No detectable unwinding activity was observed at 0°C or when the enzyme fraction was heated at 56°C for 5 min. Significant unwinding activity was observed in the broad pH range pH 7.5 to 9.5 with an optimum near 9.0. The enzyme activity was destroyed if trypsin (1 unit) was included in the reaction. The helicase reaction was totally inhibited by M13 ssDNA (100ng). Nearly 50% inhibition was observed in presence of M13 RFI DNA (200ng). Ammonium sulfate (45 mM), NaCl and KCl (200mM) were not inhibitory but potassium phosphate, pH 8.0 (100mM) was inhibitory to HDH I. (Table 3). The helicase required divalent cation for activity. Maximum activity

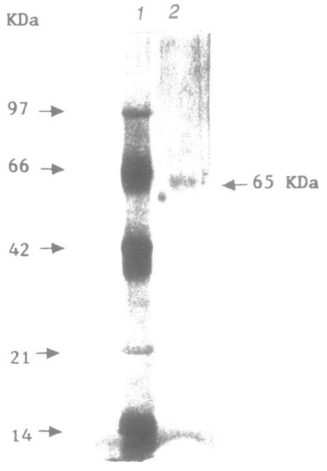

Figure 3. SDS polyacrylamide gel electrophoresis of purified fraction of HDH I. Fraction VI was separated in a 10% gel according to Laemmli (36) and stained with silver using Bio-Rad's silver stain kit. Lane 1: size markers, indicated in kilodaltons. Lane 2: purified fraction (~65ng) from ssDNA column.

Table 2 . Purification of human DNA helicase I.

Fraction	Step	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)
	Nuclear extract	150	375	N.D.	
I	Amm. Sulfate ppt.	49	210	N.D.	
II	Bio-Rex 70	142	30	N.D.	
III	Amm. Sulfate ppt.	8	24	53,124	2213
IV	DEAE Sephacel	20	13	41,340	3154
V	Heparin Sepharose	11	7.2	35,250	4896
VI	ssDNA Sepharose	4.5	0.11	15,000	136,362

N.D. = Not Determined

Table 3. Reaction requirements of the purified HDH I.

Reactions conditions	% Unwinding
Complete	65
- Enzyme	2
+ heated enzyme	2
- ATP 2	
+ dATP (2mM)	35
+ ATP γS (2mM)	2
+ ADP (2mM)	2
+ AMP (2mM)	2
+ C,G or UTP (2mM)	2
+ dC,dG or dTTP (2mM)	2
- $MgCl_2$	2
+ $MgSO_4$ (2mM)	60
+ MgOAc (2mM)	60
+ $CaCl_2$ (2mM)	12
+ $MnCl_2$ (2mM)	30
+ $ZnCl_2$ (2mM)	2
+ $CuCl_2$ (2mM)	2
Complete	
+ $(NH_4)_2SO_4$ (45mM)	58
+ KCl or NaCl (200mM)	55
+ KPO_4 (pH8,100 mM)	21
+ EDTA (5 mM)	2
+ M13 ss DNA (100 ng)	2
+ M13 RF1 DNA (200 ng)	30
+ Trypsin (1 unit)	2

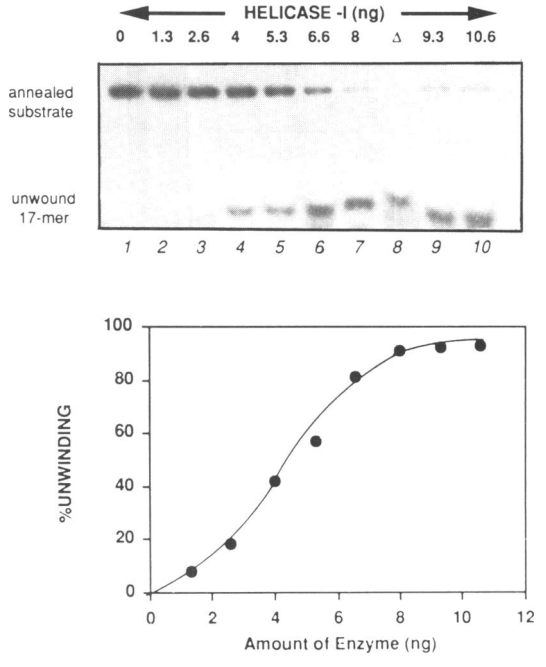

Figure 4. Titration of amount of HDH I required for maximal helicase activity. Top panel shows the autoradiographical evaluation of the experiment. Amount of HDH I is indicated at the top of each lane. Lane 1 and 8 are native and heat denatured substrate. Bottom, the unwound DNA was quantitated as described in 'Materials and Methods' and plotted as a function of the amount of HDH I used.

was obtained with Mg^{2+} [$Mg^{2+} > Mn^{2+} > Ca^{2+}$]; Cu^{2+} and Zn^{2+} were inactive (Table 3). The helicase reaction required the presence of ATP as cofactor. Although dATP supported unwinding at about half the efficiency of ATP, other NTPs failed to support any detectable unwinding; ADP and AMP were also not effective (Table 3). The nonhydrolyzable ATP analogue ATPγS was not capable of serving as cofactor for the helicase

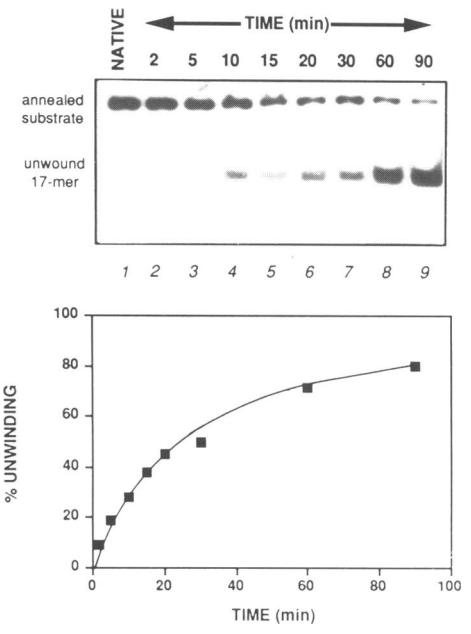

Figure 5. Kinetics of the HDH I reaction. Top panel shows the autoradiographical evaluation of the experiment. Lane 1, standard reaction without enzyme.Lanes 2–9 are helicase reaction with 5 ng of HDH I at 2, 5, 10, 15, 20, 30, 60 and 90 min. Bottom panel shows the% unwinding plotted as a function of time.

activity, suggesting that hydrolysis of ATP is essential for the enzyme to exhibit DNA helicase activity. The optimum concentration of ATP for helicase activity is around 3mM. HDH I helicase activity shows an S-shaped dependence on enzyme concentration in our assay conditions; a maximum value of 95% unwinding was observed (Fig. 4). The overall unwinding reaction using 5 ng of HDH I is approximately linear for 20 min and reaches 80% completion in 90 min (Fig. 5).

DNA Helicase activity on various substrates

Fig. 6 shows the activity of HDH I activity with different types of substrates. HDH I can unwind the partially duplex substrate whether the substrate has hanging tails at both 5′ and 3′ end (Fig. 6 A) or if it has a tail at only one end (Fig. 6 B and C) or if it has no tail (Fig. 6 E and F). In Fig. 6 D a linear substrate was used which was made by SmaI digestion of the substrate which has 5′ and 3′ end hanging tails. The results show that HDH I can also work on linear substrate and open the double helix. HDH I failed to unwind the blunt ended duplex DNA substrate shown in Fig. 6 H. Also, if the substrate has very little (8–11 bases) ss DNA as shown in Fig. 6 G, the HDH I can not unwind it.

Direction of unwinding by HDH I

Since the circular partial duplex substrate routinely used as helicase substrate does not allow for the determination of helicase directionality, we have prepared a linear substrate, as shown in Fig. 7, which contains a long linear ss DNA strand bearing short stretches of duplex DNA at both ends. On this substrate, HDH I should first bind to the internal ss region and then move through the duplex region. Unwinding in a 5′ to 3′ (with respect to the ss DNA partner) direction should result in the release of the radioactive 28-mer while unwinding in a 3′ to 5′ direction should cause release of the radioactive 15-mer from the substrate. HDH I unwinds only the 15-mer only and does not unwind the 28-mer

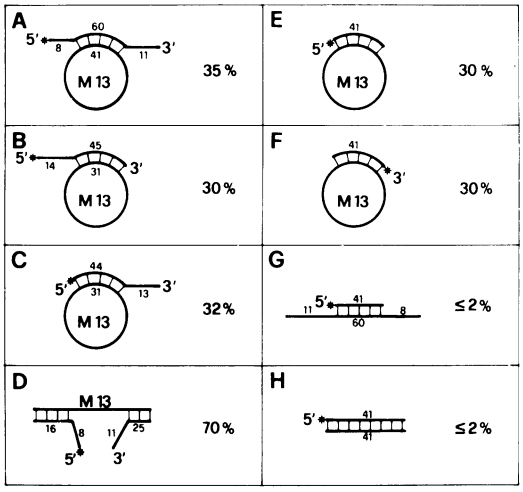

Figure 6. Helicase reaction with various substrates. The star indicates the labelled end. 5 ng of pure enzyme were present in all experiments. The% of unwinding in 20 min. is reported on the right hand side of each panel.

from the substrate (Fig. 7, lower panel), showing that HDH I moves unidirectionally in a 3′ to 5′ direction along the DNA strand to which it binds. The nuclear extract instead contains activity which unwinds both 28-mer and 15-mer from this substrate, indicating that HeLa cells also have a helicase which unwinds in the 5′ to 3′ direction that we are now further investigating (Fig. 7).

RNA-DNA unwinding by HDH I

An RNA-DNA hybrid substrate consisting of 1 kb RNA annealed in the middle with radioactively labelled complementary 18-mer (DNA) was prepared as in Fig. 8. Products of an unwinding reaction are resolved by electrophoresis in a 12% nondenaturing polyacrylamide gel and detected by autoradiography. When HDH I was incubated with this substrate in the same conditions used for the DNA helicase assay, the unwinding of the RNA-DNA hybrid was observed. (Fig. 8).

DISCUSSION

The essential role played by some helicase in opening the DNA duplex at the replication origin and in favouring the advancement of the growing fork has prompted our investigation on the number and properties of DNA helicases in cultured human cells. In previous years our group had already isolated from HeLa cells a DNA dependent ATPase capable of performing a limited unwinding of duplexes by moving along a single strand in the 5′ to 3′ direction (17, 18). This is the opposite of the polarity of HDH I; further work (see below) may show whether helicase IV, which has the same polarity as the previously described enzyme, corresponds to it. We have now initiated a systematic survey of DNA helicases from the same cells and we have thus identified four different molecular species, as judged from the different behaviour in protein fractionation. The relative abundance of each helicase appears to be rather low, so that from 100 grams of cultured cells we could isolate only 110 μg of possibly pure helicase I. A preliminary characterization of this molecular species showed that this enzyme utilizes only ATP or dATP for its reaction, is able to unwind duplex stretches present in the context of a single strand molecule, does not need a fork-

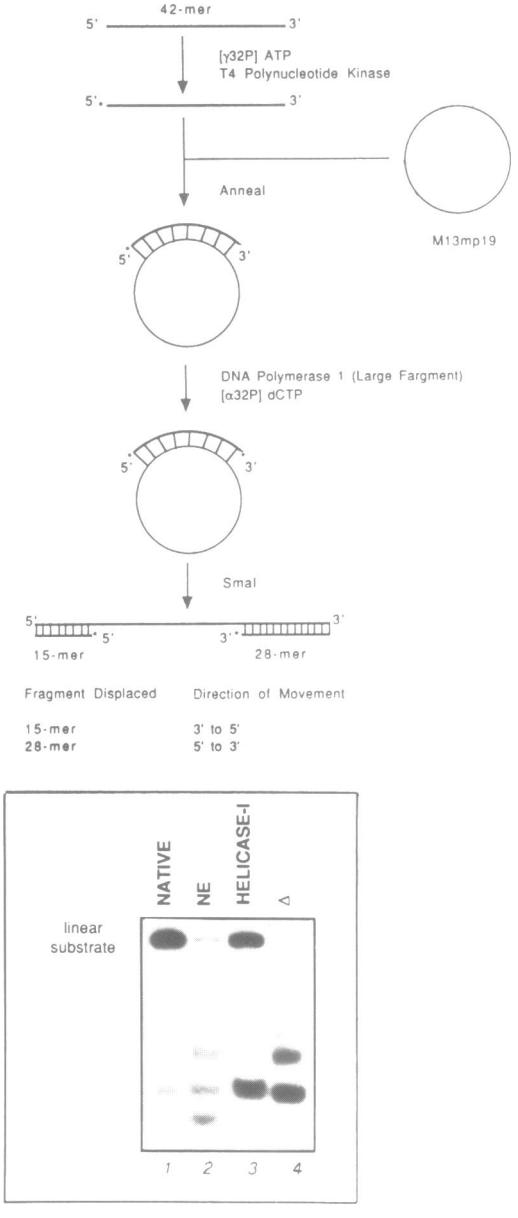

Figure 7. Directionality of unwinding by HDH I. The construction of the linear partial duplex substrate used to determine the direction of the HDH I is shown in the upper panel (see also 'Materials and Methods'). The lower panel shows the autoradiogram of the gel. Lanes 1 and 4 are the native and heat denatured substrate respectively. Lane 2: reaction with crude nuclear extract (1ug) and lane 3 purified HDH I fraction (5ng).

like structure for its unwinding activity and moves along the single strand in the 3′ to 5′ direction. This preliminary characterization does not allow yet insights on the mechanism of action or on the *in vivo* function of the enzyme, still a few comparative comments with the other known DNA helicase are warranted.

HDH I can utilize ATP or dATP only for its unwinding activity, whereas mouse helicase can use all NTPs (16) and the SV40 T-antigen uses also dTTP (9). Mg^{2+} is essential for the activity of our enzyme, and Mn^{2+} can replace it only partially, similar to T-antigen (9), calf thymus (15), mouse (16) and *X. laevis* (13) helicases. HDH I is 90% active in the presence of 200mM KCl or NaCl, whereas calf thymus (15) and T-antigen (9) helicases are inhibited at that concentration. Phosphate ions

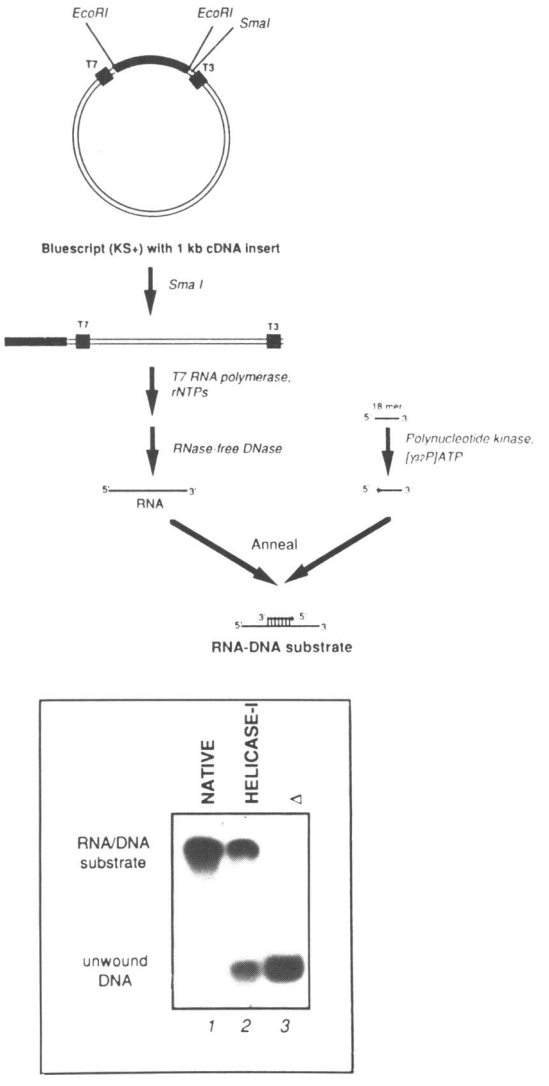

Figure 8. RNA-DNA unwinding by HDH I. The construction of the RNA-DNA substrate is shown in the upper panel (see also 'Materials and Methods'). The lower panel shows the autoradiogram of the gel. Lanes 1 and 3 are native and heat denatured substrate respectively. Lane 2 is the reaction with purified HDH I (5ng).

are inhibitory for HDH I as well as SV40 T-antigen (9), but not for *X. laevis* helicases (13).

HDH I also unwinds RNA-DNA hybrids consisting of an oligodeoxynucleotide annealed to a long stretch of RNA. This means that the helicase can bind indifferently to an RNA or DNA strand and move along it to open the duplex. Among the known DNA helicases only human p68, a conserved nuclear protein (29), *E. coli* helicase II (27) and SV40 T-antigen (30) were shown able to unwind RNA-DNA hybrids.

All prokaryotic DNA helicases which have been demonstrated, by genetic and molecular data, to be involved with the advancement of the growing fork, [i.e. the ones coded by bacteriophages T4 (3) and T7 (2) as well as by *E. coli* (1)] show a 5′ to 3′ polarity of movement: they are in fact envisaged to sit on the lagging strand, and move along with the replisome-primosome complex, unwinding DNA in front of the incoming replicating DNA polymerase on the leading strand, and preparing a suitable substrate for primase on the lagging strand. In eukaryotic systems only in two cases a function in replication has been assigned to a DNA helicase: in herpes simplex virus, a helicase-primase complex appears to move along the growing fork unwinding in the 5′ to 3′ direction in a way similar to what has been observed in prokaryots (40). In the Papova viruses (SV40 and Polyoma) the T-antigen has been shown to be essential for activation of origin, and to have a significant DNA helicase activity, non dissociable from the origin activation activity and moving in the 3′ to 5′ direction, that is opposite to the one observed in the other prokaryotic replicative helicases as well as in the herpes simplex case. The T-antigen helicase appears to have a role also in fork advancement, after origin activation (8, 41).

It is not possible to establish whether the peculiar polarity of the T antigen helicase represents a feature specific of the Papova viruses, or it corresponds to the mode of activation of eukaryotic origins in general. Much more careful and in depth studies on the *in vivo* function of HDH I will be necessary to establish whether the fact that it has the same polarity of movement as T antigen has also any functional meaning.

Among the other three DNA helicases that we have identified so far in human cells, HDH II and III appear to have the same polarity as HDH I, namely 3′ to 5′, whereas the ammonium sulfate supernatant of fraction I, containing HDH IV, shows activity moving in the 5′ to 3′ direction (to be published). We are in no position at present to ascribe any specific role to any of the forms we have identified so far, whether it be replication, repair, recombination, or some aspects of RNA metabolism, (considering that at least HDH I appears to be also an RNA-DNA helicase). More extensive purification and characterization of the HDHs described so far, and their possible production in substantial quantities by cloning, will be necessary before we can hope to establish their precise functions and their similarity, if any, with the DNA helicases already demonstrated in other eukaryotic systems.

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REFERENCES

1. Geider, K. and Hoffmann-Berling, H. (1981) *Ann. Rev. Biochem.* **50**, 233–260.
2. Matson, S. W., Tabor, S. and Richardson, C. C. (1983) *J. Biol. Chem.* **258**, 14017–14024.
3. Liu, C. C. and Alberts, B. M. (1981) *J. Biol. Chem.* **256**, 2813–2820.
4. Abdel-Monem, M. and Hoffmann-Berling, H. (1976) *Eur. J. Biochem.* **65**, 441–449.
5. Matson, S. W. and George, J. W. (1987) *J. Biol. Chem.* **262**, 2066–2076.
6. Lee, M. S. and Marians, K. J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8345–8349.
7. Wood, E. R. and Matson, S. W. (1987) *J. Biol. Chem.* **262**, 15269–15276.
8. Stahl, H., Droge, P. and Knippers, R. (1986) *EMBO J.* **5**, 1939–1944.
9. Goetz, G. S., Dean, F.B., Hurwitz, J. and Matson, S. W. (1988) *J. Biol. Chem.* **263**, 383–392.
10. Sugino, A., Ryu, B. H., Sugino, T., Naumovski, L. and Friedberg, E. C. (1986) *J. Biol. Chem.* **261**, 11744–11750.
11. Sung, P., Prakash, L., Matson, S. W. and Prakash, S. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8951–8955.

12. Hotta, Y. and Stern, H. (1978) *Biochemistry* **17**, 1872–1880.
13. Poll, E. H. A. and Benbow, R. M. (1988) *Biochemistry* **27**, 8701–8706.
14. Hubscher, U. and Stalder, H. P. (1985) *Nucleic Acids Res.* **13**, 5471–5483.
15. Thommes, P. and Hubscher, U., (1990) *J. Biol. Chem.* **265** (In Press).
16. Seki, M., Enomoto, T., Hanaoka, F. and Yamada, M. (1987) *Biochemistry* **26**, 2924–2928.
17. Cobianchi, F., Riva, S., Mastromei, G., Spadari, S., Pedrari-Noy, G. and Falaschi A. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 639–647.
18. Falaschi, A., Cobianchi, F. and Riva, S. (1980) *Trends in Biochemical Sciences*, **June** 154–157.
19. Cobianchi, F., Biamonti, G., Mastromei, G., Falaschi, A. and Riva, S. (1982) *Biochem. Biophys. Res. Commun.* **104**, 402–409.
20. Abdel-Monem, M., Taucher-Scholz, G. and Klinkert, M. Q. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4659–4663.
21. Husain, I., VanHouten, B., Thomas, D. C., Abdel-Monem, M. and Sancar, A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6774–6778.
22. Lu, A.-L., Welsh, K., Clark, S., S.-S. and Modrich, P. (1984) *Cold Spring Harbor Symp. Quant. Biol.* **49**, 589–596.
23. LeBowitz, J. H. and McMacken, R. (1986) *J. Biol. Chem.* **261**, 4738–4748.
24. Cox, M. M. and Lehman, I. R. (1987) *Annu. Rev. Biochem.* **56**, 229–262.
25. Scott, J. F. and Kornberg, A. (1978) *J. Biol. Chem.* **253**, 3292–3297.
26. Brennan, C. A., Dombroski, A. J. and Platt, T. (1987) *Cell* **48**, 945–952.
27. Matson, S. W. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4430–4434.
28. Ray, B. K., Lawson, T. G., Kramer, J. C., Cladaras, M. H., Grifo, J. A., Abramson, R. D., Merrick, W. C., and Thach, R. E. (1985) *J. Biol. Chem.* **260**, 7651–7658.
29. Hirling, H., Scheffnef, M., Restle, T. and Stahl, H. (1989) *Nature* **339**, 562–564.
30. Scheffner, M., Knippers, R. and Stahl, H. (1989) *Cell* **57**, 955–963.
31. Hodgman, T. C. (1988) *Nature* **333**, 22–23.
32. Linder, P., Lasko, P.F., Leroy, P., Nielsen, P. J., Nishi, K., Schnier, J. and Slonimski, P. (1989) *Nature* **337**, 121–122.
33. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
34. Tuteja, N., Danciger, M., Klisak, I., Tuteja, R., Inana, G., Mohandas, T., Sparkes, R. S. and Farber, D. B. (1990) *Gene* **88**, 227–232.
35. Kaiserman, H. B., Ingebritsen, T. S. and Benbow, R. M. (1988) *Biochemistry* **27**, 3216–3222.
36. Hughes, M. J., Liang, H., Jiricny, J. and Jost, J.-P. (1989) *Biochemistry* **28**, 9137–9142.
37. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
38. Laemmli, U. K. (1970) *Nature* **227**, 680–685.
39. Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983) *Nucleic Acid Research* **11**, 1475–1489.
40. Crute, J. J., Tsurumi, T., Zhu, L., Weller, S. K., Olivo, P. D., Challberg, M. D., Mocarski, E. S. and Lehman, I. R. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2186–2189.
41. Stahl, H. and Knippers, R. (1987) *Biochim. Biophys. Acta* **910**, 1–10.