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# DNA helicase III from HeLa cells: an enzyme that acts preferentially on partially unwound DNA duplexes

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## ABSTRACT

**Human DNA helicase III, a novel DNA unwinding enzyme, has been purified to apparent homogeneity from nuclear extracts of HeLa cells and characterized. The activity was measured by using a strand displacement assay with a $^{32}P$ labeled oligonucleotide annealed to M13 ssDNA. From 305 grams of cultured cells 0.26 mg of pure protein was isolated which was free of DNA topoisomerase, ligase, nicking and nuclease activities. The apparent molecular weight is 46 kDa on SDS polyacrylamide gel electrophoresis. The enzyme shows also DNA dependent ATPase activity and moves unidirectionally along the bound strand in 3′ to 5′ direction. It prefers ATP to dATP as a cofactor and requires a divalent cation ($Mg^{2+} > Mn^{2+}$). Helicase III cannot unwind either blunt-ended duplex DNA or DNA-RNA hybrids and requires more than 84 bases of ssDNA in order to exert its unwinding activity. This enzyme is unique among human helicases as it requires a fork-like structure on the substrate for maximum activity, contrary to the previously described human DNA helicases I and IV, (Tuteja et al. Nucleic Acids Res. 18, 6785–6792, 1990; Tuteja et al. Nucleic Acids Res. 19, 3613–3618, 1991).**

## INTRODUCTION

Many processes in nucleic acid metabolism such as replication, repair, recombination and transcription need single-stranded (ss) DNA as a substrate for DNA and RNA polymerases (1). The family of enzymes responsible for providing the ssDNA during these processes by unwinding the duplex are known as DNA helicases. This is an energy requiring process driven by the hydrolysis of the γ-phosphate of a nucleoside—or deoxy-nucleoside 5′-triphosphate (2). In fact all the known DNA helicases have been found to contain intrinsic DNA-dependent ATPase activity (2–4).

DNA helicases were discovered for the first time in 1976 in *E.coli* (5). They are ubiquitous in both prokaryotes and eukaryotes (3,4). DNA helicases usually bind to one strand of the DNA and move along it unidirectionally in either 3′ to 5′ or 5′ to 3′ direction and thus displace the other strand (3). However, in contrast to most other helicases, the recBCD helicase of *E.coli* interacts with, and translocates on, both strands of the DNA from the ends, suggesting an absence of, or undefined polarity (6). Most of the DNA helicases need ssDNA adjacent to the duplex region to be unwound, with the notable exception of the SV40 T antigen (7), *E.coli* helicase II (8) and *E.coli* RecQ (9) which can also unwind fully duplex linear DNA.

Apart from DNA helicases, RNA-RNA and RNA-DNA helicases have also been identified and these have been considered to function in transcription (10), translation (11) or RNA splicing (12). The *E.coli* transcription termination factor rho is a DNA-RNA helicase which presumably facilitates the release of RNA transcripts from the DNA template (13). In prokaryotes, also DNA helicase II of *E.coli* (14) unwinds DNA-RNA hybrids. In animal systems both the SV40 T antigen (7) and calf thymus helicase II (15) can unwind DNA-RNA as well as RNA-RNA hybrids. Several other putative helicases have been proposed in animal systems on the basis of their primary structures (16,17).

Much less is known about the DNA helicases present in human cells. We have reported earlier (18) the existence of at least four different human DNA helicases, namely HDH I, II, III (based on the differential chromatographic fractionation of the proteins present in a 35% w/v ammonium sulfate precipitate) and HDH IV (present in the ammonium sulfate supernatant). We have already purified to homogeneity and characterized HDH I and HDH IV (18,19). HDH I is a 65 kDa protein, which moves in 3′ to 5′ direction along the bound strand and unwinds also RNA-DNA hybrid (18) whereas HDH IV is a 100 kDa protein, moves in 5′ to 3′ direction and also unwinds RNA-DNA hybrids (19). Recently, another DNA helicase from HeLa cells has also been described which is dependent on the homologous single stranded DNA binding protein to unwind DNA in the 3′ to 5′ direction (20).

Here we present the purification to homogeneity and characterization of human DNA helicase III from HeLa cell nuclear extract.

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## MATERIALS AND METHODS

### Cell cultures and buffers

HeLa cells were grown as previously described (18). 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. All the buffers used during purification contained 1 mM PMSF, 1 mM sodium bisulfite, 1 μM pepstatin and 1 μM leupeptin. Buffer A contained 20 mM HEPES (pH 8.0), 0.1 M NaCl, 1 mM DTT, 1 mM EDTA and 20% glycerol. Buffer B contained 50 mM Tris-HCl (pH 8.0), 0.1 M KCl, 1mM DTT, 1mM EDTA and 10% glycerol.

### DNA and RNA oligonucleotides

The DNA oligonucleotides used for making DNA helicase substrates were synthesised using an Applied Biosystems 380A DNA synthesizer. A total of 12 different oligodeoxynucleotides (17 to 101-mers) complementary or partially complementary to ss M13 DNA were used for constructing various DNA substrates. Their sequences were described previously (18,19). An RNA 17-mer complementary to M13 ssDNA was synthesised from Primm Prodotti Immunobiotecnologici, Italy. The sequence is 5'-GUUUUCCCAGUCACGAC-3'.

### Preparation of DNA helicase substrates

The DNA substrate used in the helicase assay consists of $^{32}P$ labeled complementary oligonucleotides annealed to M13mp19 phage ssDNA to create a partial duplex. A substrate with 3' and 5' hanging tails (Figure 6A) was used unless otherwise stated and prepared as follows (19). Twentyfive ng of the 47-mer oligonucleotide were 5' end labeled by T4 polynucleotide kinase (5 units) and 0.925MBq of [$^{32}P$] ATP (185TBq/mmol) in a buffer containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine and 0.1 mM EDTA at 37°C for 1 hour. This labeled oligomer was annealed to M13mp19 ssDNA (1 μg) in 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT. This mixture was heated at 95°C for 1 min., then allowed to anneal at 65°C for 20 min followed by slow cooling to room temperature and the substrate was purified by gel filtration through 1ml sepharose 4B column. Small linear substrates were prepared by labeling 25 ng of 17-mer (19) and annealing it with 100 ng of 101-mer (19) as described above. The blunt ended duplex DNA substrate was also prepared as described above using 25 ng of 17-mer and complementary 17-mer (19). DNA-RNA substrate was prepared by 5'-end labeling of RNA oligonucleotide (25 ng) as described above but in presence of one unit of RNase block. The labeled RNA oligomer was annealed with M13mp19 ssDNA (1 μg) in 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl and 5 units of RNase Block at 65°C for 1 hour and immediately cooled on ice. The unreacted nucleotides were removed by gel filtration through 1ml sepharose 4B.

### DNA helicase assay

The standard reaction mixture (10 μl) consisted of 20 mM Tris-HCl (pH 8.5), 8 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM ATP, 30mM KCl, 4% (w/v) sucrose, 80 μg/ml BSA, 1.0 ng of $^{32}P$ labeled helicase substrate (~1000 cpm) and 1 μl of helicase fraction. The reaction mixture was incubated at 37°C for 30 min and stopped by addition of 0.3% SDS, 10 mM EDTA, 5% glycerol and 0.03% bromophenol blue. After further incubation at 37°C for 5 min the substrate and product were separated by electrophoresis on a 12% native polyacrylamide gel (mini gel size, 8×10cm) in TBE buffer (89 mM Tris-borate, pH 8.2 and 2 mM EDTA). After electrophoresis the gel was fixed in a 10% methanol and 10% acetic acid solution for 10 min. The gel was dried and exposed to Amersham Hyperfilm for autoradiography with an intensifying screen at −80°C overnight. DNA unwinding was quantitated by excising the radioactive bands from the gel and counting in Beckman liquid scintillation fluid. One unit of helicase activity is defined as the amount of enzyme that unwinds 30% of the DNA helicase substrate at 37°C in 30 min (1% in one min.) in the linear range of enzyme concentration dependence. The helicase activity using DNA-RNA substrate was also determined as described above but in presence of 1 unit of RNase Block.

### DNA dependent ATPase assay

The standard reaction mixture (10 μl) contained 20 mM Tris-HCl (pH 8.5), 8mM dithiothreitol, 1 mM $MgCl_2$, 30 mM KCl, 4% (w/v) sucrose, 80 μg/ml BSA, 1 mM ATP, 1665Bq [$^{32}P$] ATP (185TBq/mmol), 1 μg ssDNA from HeLa Cells and 100 ng pure HDH III. Assay was performed both in presence or absence of ssDNA. The mixture was incubated for 30 min at 37°C and the reaction was stopped by chilling to 0°C. One μl of the mixture was spotted onto a polyethyleneimine-cellulose thin-layer strip (0.6×6cm), and ascending chromatography was performed in 0.5 M LiCl, 1 M formic acid at room temperature for about 15 min. The strip was dried at room temperature and exposed to Amersham hyperfilm to identify the radioactive spots of ATP and Pi. For quantitation these spots were cut from the strip and counted with Beckman liquid scintillation fluid.

### Preparation of ssDNA sepharose

The genomic DNA from HeLa cells was isolated as described by Sambrook et al. (21). The DNA obtained was subjected to ultrasonication by 15 pulses of 30 seconds each at 60 watt to an optimum size of 0.50 to 3.0 Kb as checked by agarose gel electrophoresis. The sonicated DNA was denatured in 0.2 M NaOH and ethanol precipitated. The resulting ssDNA was coupled to cyanogen bromide activated sepharose 4B as described in the Pharmacia booklet. The efficiency of coupling was found to be 1mg ssDNA per ml of packed sepharose 4B.

### Miscellaneous

DNA topoisomerases were assayed as described by Kaiserman et al. (22), except that the plasmid DNA used was Bluescript containing a cDNA insert (1kb) of human cGMP phosphodiesterase (23). DNA ligase activity was checked as described earlier (18) and DNA nicking activity was assayed as described by Hughes et al. (24). Proteins were resolved by SDS-polyacrylamide gel (10%) electrophoresis (25), silver staining was done using a Bio Rad kit. Protein concentration determination was performed using a Bradford protein assay kit (Bio Rad).

## RESULTS

### Purification of human DNA helicase III (HDH III)

The substrate used for the purification procedure and for the characterization contained both 5' end and 3' end hanging tails of 15 nucleotides each on 17 nucleotides annealed to M13mp19 ssDNA (Figure 6A). The helicase activity was measured as reported in Materials and Methods.

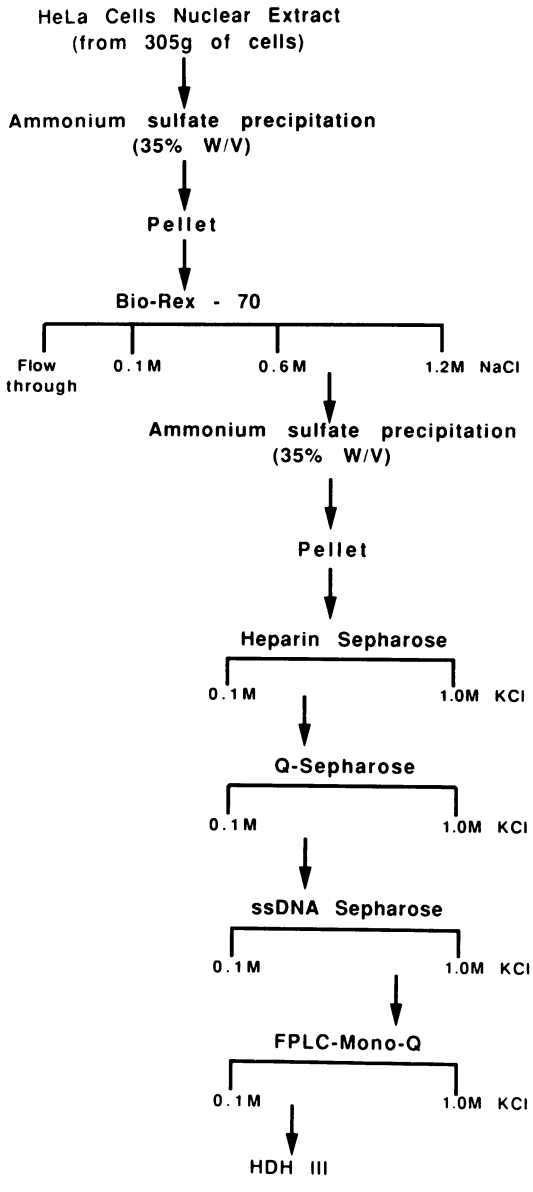


Figure 1. Purification scheme for HDH III. The strategy used for fractionation of HDH III from HeLa cells nuclear extract is shown. The numbers shown indicate the molarity of the NaCl or KCl gradient used to elute HDH III activity.

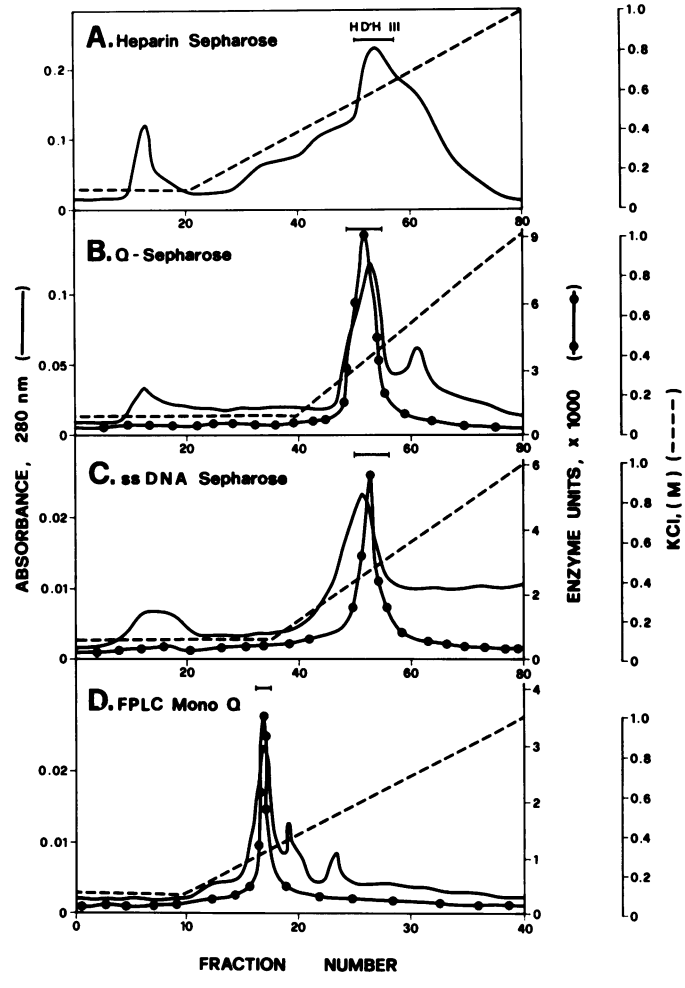


Figure 2. The protein elution and corresponding helicase activity profiles on Heparin Sepharose (A), Q-Sepharose (B), ssDNA-Sepharose (C) and FPLC Mono Q (D) columns are shown. The molar range of salt used to elute each column is indicated in the right ordinate axis. The detailed description of the chromatographic procedures is given in the text. The pooled fractions are indicated by the horizontal bars.

A summary of the procedure used for purification of HDH III is outlined in Figure 1. All the operations were carried out at 0–4°C and, in all chromatographic steps, protein elution was monitored continuously by measuring the absorption at 280 nm. As a first step, nuclear extract was prepared from 305 grams of frozen HeLa cells as described previously (26) followed by precipitation of proteins by adding ammonium sulfate (0.35 g/ml) with constant stirring. The mixture was stirred for an additional hour and the precipitate was collected by centrifugation (27,000×$g$, 20 min.). The supernatant containing HDH IV activity was removed (19), the pellet resuspended and dialyzed in buffer A (fraction I: 455 ml).

Fraction I was loaded onto a 169 ml Bio-Rex 70 column (2.3×32 cm) which was pre-equilibrated with buffer A. The column was washed with 3 column volumes of buffer A. The flow-through protein fractions contained the HDH I activity purified by us earlier (18). The bound proteins were eluted with 5 column volumes of a linear gradient between 0.1 and 0.6 M NaCl in buffer A followed by a second linear gradient of 4 column volumes ranging from 0.6 M to 1.2 M NaCl in buffer A. The protein fractions of the first gradient contained HDH II as described by us earlier [(18), purification now in progress]. The active fractions from the second gradient were eluted around 0.8 M NaCl in buffer A (fraction II, 150 ml).

The proteins of fraction II were precipitated by ammonium sulfate (0.35 g/ml) as described above. The pellet was resuspended and dialyzed in buffer B (fraction III, 25 ml). The amount of helicase activity present in fraction I to III could not be determined precisely due to the presence of nucleases.

The next step in the purification process involved fractionation by heparin sepharose chromatography (Figure 2A). Fraction III was applied onto a 19 ml heparin sepharose column (1.6×9 cm) equilibrated with buffer B. The column was washed with 3 column volumes of buffer B and the proteins were eluted with a 7 column volumes linear gradient from 0.1 M to 1.0 M KCl in buffer B. The peak fractions containing helicase activity eluted at about 0.57 M KCl and were pooled (fraction IV; 7.7 ml,

**Table I.** Purification of human DNA helicase III (HDH III)

| Fraction | Step | Volume (ml) | Protein (mg) | DNA helicase activity Total (units) | Specific (units/mg) |
|---|---|---|---|---|---|
| | Nuclear Extract | 480 | 2100 | N.D. | |
| I | Amm. Sulfate ppt. | 455 | 910 | N.D. | |
| II | Bio-Rex 70 | 150 | 111 | N.D. | |
| III | Amm. Sulfate ppt. | 25 | 50 | N.D. | |
| IV | Heparin Sepharose | 7.7 | 15 | 29,300 | 1,950 |
| V | Q-sepharose | 15 | 5 | 24,900 | 4,980 |
| VI | ssDNA sepharose | 10 | 2.3 | 13,300 | 5,800 |
| VII | FPLC-Mono Q | 3.5 | 0.260 | 10,500 | 40,400 |

N.D. = Not Determined

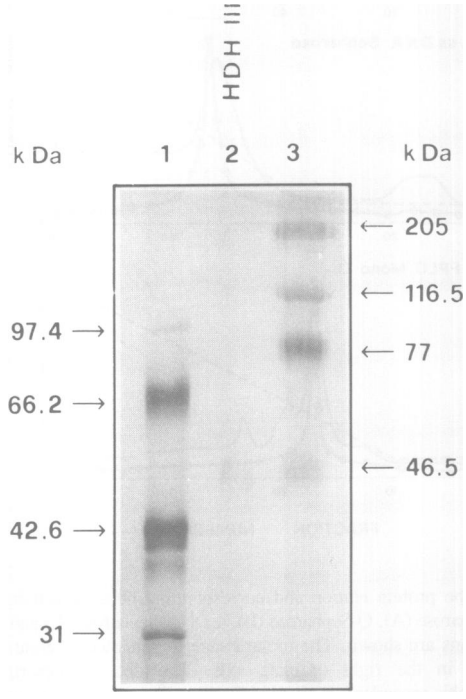


**Figure 3.** SDS polyacrylamide gel electrophoresis of purified HDH III. Fraction VII was separated in a 10% gel according to Laemmli and stained with silver using a Bio-Rad silver stain kit. Lanes 1 and 3: size markers, indicated in kDa. Lane 2: purified HDH III (fraction VII, ~ 0.2 μg) from FPLC Mono Q column.

**Table II.** Reaction requirements of the purified HDH III activity[a]

| Reaction Conditions | % Unwinding |
|---|---|
| Complete | 70 |
| − Enzyme | < 2 |
| + heated enzyme (56°×5min) | < 2 |
| − ATP | < 2 |
| " + dATP (1mM) | 60 |
| " + ATP γS (1mM) | < 2 |
| " + ADP (1mM) | < 2 |
| " + AMP (1mM) | < 2 |
| " + C, G or UTP (1mM) | < 2 |
| " + dC, dG or dTTP (1mM) | < 2 |
| − $MgCl_2$ | < 2 |
| " + $MgSO_4$ (1mM) | 70 |
| " + $Mg(C_2H_3O_2)_2$ (1mM) | 71 |
| " + $CaCl_2$ (1mM) | < 2 |
| " + $MnCl_2$ (1mM) | 60 |
| " + CdCl2 | < 2 |
| " + $CoCl_2$ | < 2 |
| " + $ZnSO_4$ (1mM) | < 2 |
| " + $CuCl_2$ (1mM) | < 2 |
| " + $NiCl_2$ (1mM) | < 2 |
| " + $AgNO_3$ (1mM) | < 2 |
| + $(NH_4)_2SO_4$ (45mM) | 65 |
| + KCl or NaCl (300mM) | < 2 |
| + $KPO_4$ (pH8, 100mM) | 50 |
| + EDTA (5mM) | < 2 |
| + M13 ssDNA (30μM as P) | < 2 |
| + M13 RFI DNA (30μM as P) | 70 |
| + E.coli t-RNA (30μM as P) | 71 |
| + Trypsin (1 unit) | < 2 |
| + Poly [A], [C] or [U](30μM as P) | 69 |
| + Poly [G] (30mM as P) | 35 |
| + Sarcosyl (0.04%) | < 2 |

[a] Helicase reaction was carried out with 100 ng of pure protein (fraction VII) using hanging tails substrate as described under 'Materials and Methods'.

29,300 units). At this point we noticed that also the shoulders on either side of the peak fractions were active regardless of whether the substrates had hanging tails or not, whereas the peak fractions showed helicase activity only with substrate containing hanging tails and was almost inactive otherwise. For this purification only the peak fractions, strictly requiring a hanging tail substrate, were pooled for further processing.

Fraction IV was first diluted to 44 ml with buffer B and loaded onto a 10 ml Q-sepharose fast flow column (1.6×5.1 cm) equilibrated with buffer B (Figure 2B). The column was washed with 3 column volumes of buffer B and eluted with 6 column volumes of a linear gradient from 0.1 M to 1.0 M KCl in buffer B. The activity eluted from the column at about 0.40 M KCl in buffer B (fraction V; 15 ml, 24,900 units).

Fraction V was diluted to 45 ml with buffer B, made 1 mM in $MgCl_2$ and loaded onto a 4 ml ssDNA-sepharose 4B column (1.6×2 cm) equilibrated with buffer B containing 1 mM $MgCl_2$. The ssDNA-sepharose 4B was prepared by coupling ssDNA from HeLa cells to cyanogen bromide activated sepharose 4B as described in 'Materials and Methods'. The column was washed with 5 column volumes of buffer B containing 1 mM $MgCl_2$ and eluted with linear gradient from 0.1 M to 1.0 M KCl in the same buffer. The helicase activity eluted from the column at about 0.70 M KCl (Figure 2C). The active fractions were pooled for further purification (fraction VI; 10 ml, 13,300 units).

Fraction VI was diluted with buffer B and loaded onto an 8 ml FPLC Mono Q (HR 10/10) column equilibrated with buffer B. The column was washed with buffer B and the proteins were eluted with 3 column volumes of linear gradient from 0.1 M to

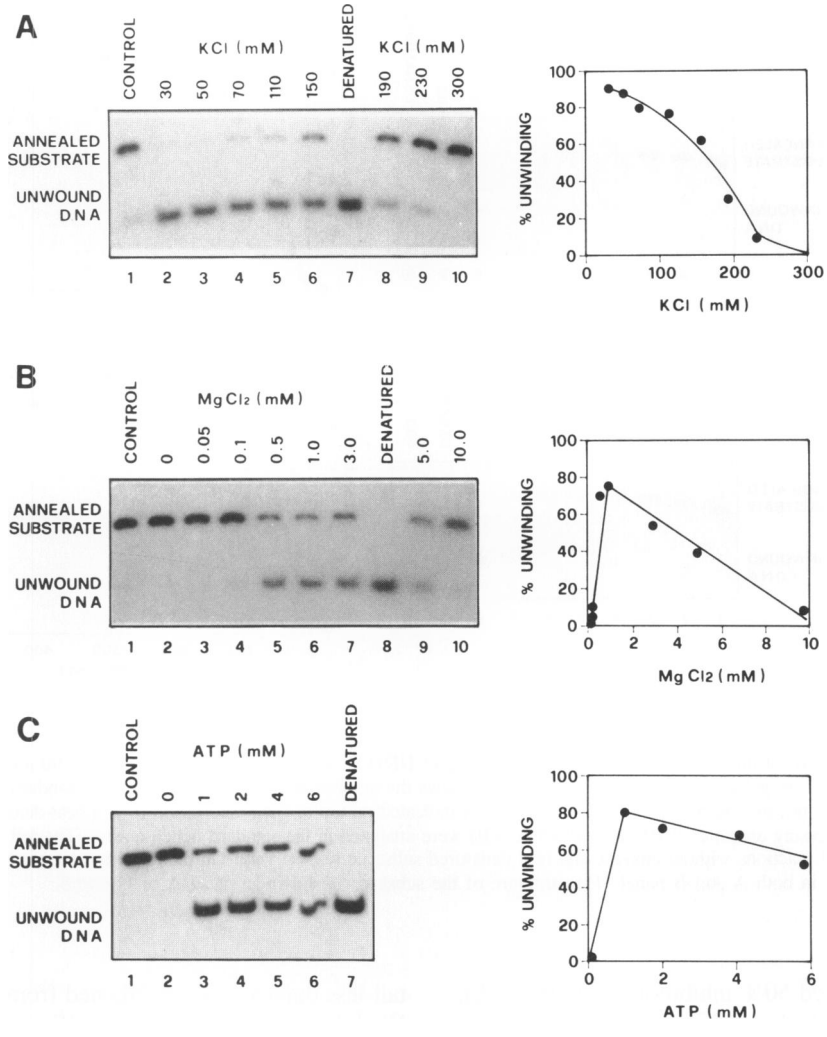


**Figure 4.** Effect of KCl (A), $MgCl_2$ (B) and ATP (C) concentration on the activity of 100 ng of pure DNA helicase (fraction VII). The structure of the substrate is shown in panel A of Figure 6. On the left side of each panel the autoradiogram of the gel is shown. The 'control' lane corresponds to a reaction without enzyme and the 'denatured' lane to the substrate heated 2 min. at 95°C. On the right side of each panel, the unwound DNA was quantitated as described in Materials and Methods and plotted. The activity is shown as% unwinding.

1 M KCl in buffer B. The helicase activity eluted at 0.27 M KCl (fraction VII; 3.5 ml, 10,500 units). The elution profile of the proteins is shown in Figure 2D.

A summary of the purification is shown in Table I. Fraction VII showed one band of 46 kDa on SDS-polyacrylamide gel electrophoresis in silver stain (Figure 3, lane 2). Overall, from 305 g of HeLa cells we were able to obtain 0.26 mg of apparently pure enzyme with specific activity of 40,400 units/mg. The purified HDH III was free of nucleases, topoisomerases, DNA ligase or DNA nicking activities.

DNA dependent ATPase activity was present at a level of 18 pmol of ATP hydrolysed in 30 min. per 100 ng of pure HDH III.

## Reactions requirements and characterization of HDH III

The best temperature for the helicase reaction is 37°C and the best pH range is 8 to 9 (data not shown). Little activity (25%) was observed at 25° and no detectable unwinding activity was found at 0°C or when the enzyme was pre-heated at 56°C for 5 min. Table II shows the reaction requirements of HDH III. The enzyme activity was destroyed if trypsin (1 unit) or sarcosyl (0.04%) was included in the reaction. The helicase reaction was inhibited by 100 fold excess of M13 ssDNA (30 $\mu$M as P), or by EDTA (5 mM) whereas M13 RFI DNA and *E.coli* tRNA in the same excess have no effect.

For maximum activity, HDH III requires 30 mM KCl (Figure 4A), 1 mM $MgCl_2$ (Figure 4B) and 1 mM ATP (Figure 4C). KCl at 300 mM or $MgCl_2$ at 10 mM were completely inhibitory to HDH III activity. ATP at 15 mM totally inhibits the enzyme reaction (data not shown). The helicase reaction required the hydrolysis of ATP since the poorly hydrolysable analog ATPγS was inactive as a cofactor. Compared to ATP other nucleoside 5'-triphosphates, AMP and ADP were inactive, with the exception of dATP which supported more than 85% of the activity (Table 2). $Mg^{2+}$ is required for maximum activity as compared to $Mn^{2+}$ which showed 85% of activity. All the other tested cations such as $Ca^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Ni^{2+}$, $Ag^{2+}$, $Cd^{2+}$ and $Co^{2+}$ were not active. $MgCl_2$ can be replaced either by magnesium sulfate or magnesium acetate. Ammonium sulfate (45 mM) has almost no effect on helicase activity while potassium phosphate at 100 mM reduced the activity by 30%.

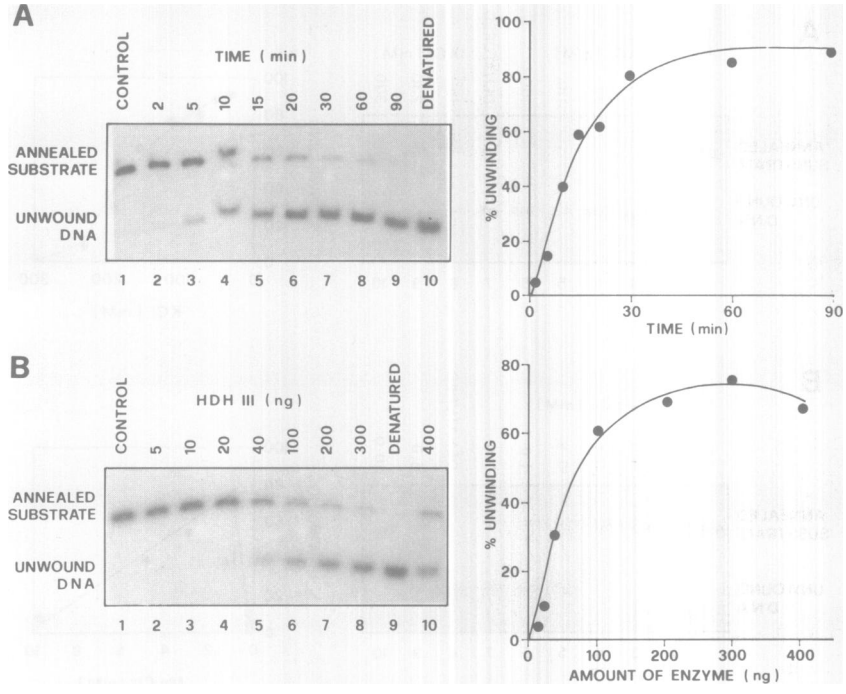


**Figure 5.** Kinetics (A) and titration (B) of human DNA helicase III. A: 100 ng of HDH III was added to a final volume of 90 μml and aliquots of 10 μl removed at various time intervals and analyzed by gel electrophoresis. The left panel shows the autoradiogram of the gel. Lane 1, standard reaction without enzyme. Lane 2 to 9 are helicase reactions at different time intervals. The time of reaction is indicated on top of each lane. Lane 10 is a heat-denatured substrate. The right panel shows the quantitative data. B: increasing amounts of HDH III (fraction VII) were analyzed in the standard helicase assay The left panel shows the autoradiogram of the gel. Lane 1 and 9 are control reactions without enzyme and heat denatured substrate respectively. On the right panel DNA was quantitated and plotted The substrate with hanging tails is used in both A and B panel. The structure of the substrate is shown in panel A of Figure 6.

Poly [G] (30 μM as P) showed 50% inhibition while Poly [A], [C] and [U] at 30 μM as P had no effect on helicase activity (Table 2).

In presence of 100 ng (4 units) of HDH III the unwinding was linear up to 10 min. and saturated at 60 min. (Figure 5A). Titration of DNA helicase activity with increasing amounts of helicase in the standard 30 min. reaction in presence of 1 ng substrate showed an approximately linear response up to 40% unwinding and a saturation at about 100 ng of HDH III (i.e. with an approximately four thousand fold excess of enzyme molecules over substrate molecules); the activity so measured levels off at 80% unwinding, even by increasing the enzyme concentration to 300 ng (Figure 5B). The DNA helicase reaction can be competed with non radioactive substrate and ss M13 DNA (see Table 2).

### DNA helicase activity on various substrates

The helicase activity was measured on different model substrates with 100 ng of pure protein. The structure of the substrates and the results obtained are shown in Figure 6. HDH III prefers a substrate with hanging tails as compared to those without. It can easily unwind a 17-mer duplex with both 5′ end and 3′ end tails (Figure 6A) as well as if the substrate has only 5′ end tail (Figure 6B) or 3′ end tail (Figure 6C). If the duplex region of hanging tails substrate increased from 17-mer to 40-mer HDH III was less efficient but still showed significant unwinding (5%, see Figure 6D). HDH III activity was reduced to one fourth if the annealed 17-mer had no hanging tail (Figure 6E). If the same tail-less duplex was lenghthened from 17-mer to 25-mer, HDH III did not show any activity (Figure 6F). HDH III failed to unwind also blunt-ended 17-mer duplex DNA substrate (Figure 6G). Also linear substrate with a single strand portion of 84 bases or less on either side of the 17-mer duplex could not be unwound by HDH III as shown in Figure 6H, I, and J.

### Direction of unwinding by HDH III

Two different substrates, one for 3′ to 5′ and one for 5′ to 3′ direction were used in order to determine the polarity of the enzyme. The substrates consisted of long linear ssDNA with short duplex ends. The construction of the substrates was described earlier (19) and their structures are shown in Figure 7. The substrate which reveals the 3′ to 5′ direction is radioactively labeled at the 5′ end of the short duplex present at the 5′ end of the long ssDNA (Figure 7A); the substrate for the 5′ to 3′ direction is labeled at 3′ end of a 17-mer on 3′ side of ssDNA (Figure 7B). HDH III unwinds unidirectionally in a 3′ to 5′ direction along the ssDNA strands to which it binds, since it showed unwinding only with the substrate specific for 3′ to 5′ direction (Figure 7A) and not with 5′ to 3′ direction substrate (Figure 7B).

### DNA-RNA unwinding by HDH III

The specific substrate for DNA-RNA unwinding consisted of a [$^{32}$P] labeled RNA 17-mer annealed to M13 ssDNA. (Figure 8). The results show that HDH III cannot unwind the DNA-RNA hybrid (lane 2 of Figure 8).

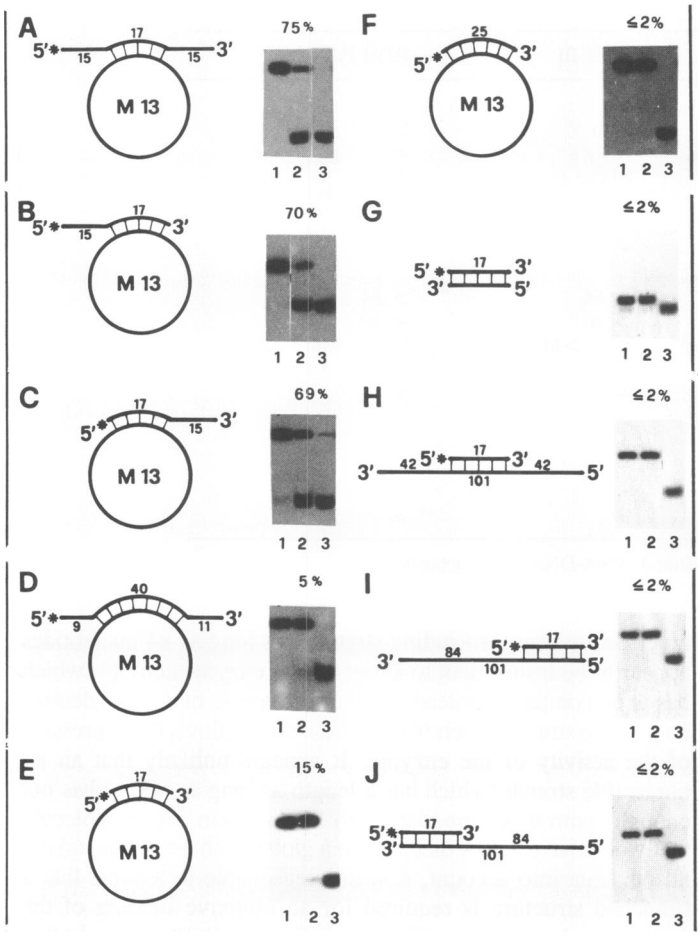


**Figure 6.** Helicase activity with various substrates. The helicase reaction was performed with 100 ng of pure HDH III (Fraction VII) as described under Materials and Methods. Each panel shows the structure of the substrate used and the autoradiogram of the gel. Asterisks denote the 32P labeled end. Percent unwinding is shown on top of each autoradiogram. Lane 1 and 3 are control reaction without enzyme and heat denatured substrate respectively. Lane 2 is reaction with pure HDH III.

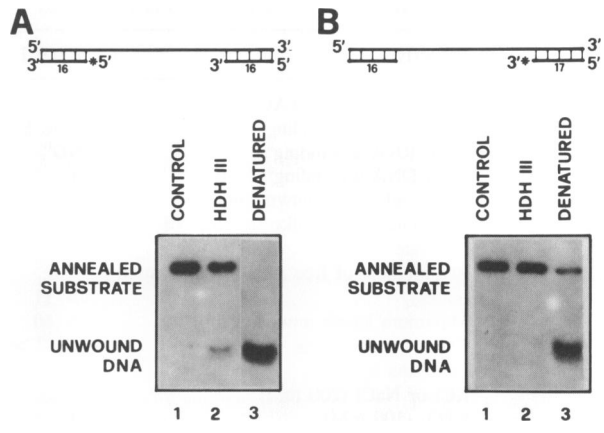


**Figure 7.** Direction of unwinding by human DNA helicase III. A: the top shows the structure of the linear substrate used for 3′ to 5′ activity and the bottom shows the autoradiogram of the gel. B: the top shows the structure of the linear substrate for the 5′ to 3′ activity and the bottom shows the autoradiogram of the gel. In each case 100 ng of HDH III (fraction VII) were used. Lane 1 and 3 are controls without enzyme and with heat-denatured substrate respectively.

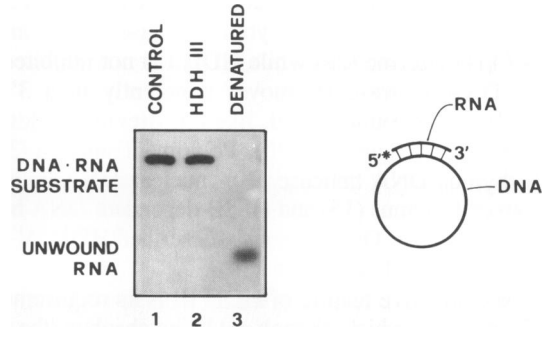


**Figure 8.** DNA-RNA unwinding by HDH III. The structure of the substrate used to determine the unwinding of a DNA-RNA hybrid is shown on the right. The construction of the substrate and the helicase assay were performed as described under Materials and Methods. The autoradiogram of the gel is shown on the left side. Lane 1 and 3 are the controls without enzyme and with heat denatured substrate, respectively.

## DISCUSSION

A novel DNA helicase, previously defined as human DNA helicase III, (18) was purified to homogeneity from nuclear extracts of HeLa cells. As a part of our systematic study of DNA helicases present in human cells we have already reported earlier the existence of at least four different DNA helicases as well as the purification of HDH I and IV (18, 19). HDH III is present in the 35% (w/v) ammonium sulfate precipitate of the nuclear extract like HDH I and II (18) and unlike HDH IV which is present in the supernatant (19). HDH III is present in low abundance: from 305 grams of cultured cells we obtained only 260 μg of pure HDH III. According to SDS-polyacrylamide protein gel electrophoresis the purified HDH III has a molecular weight of about 46 kDa as compared to 65 kDa of HDH I and 100 kDa of HDH IV. Some of the properties of the three purified HDH's are summarized in Table III.

Human DNA helicases I, II and III are fractionated on the basis of their behaviour on Bio-Rex 70 column. Helicase I does not bind while II and III bind to Bio-Rex 70. Recently, by using procedures very similar to ours, two distinct helicases from nuclei of calf thymus have been reported (15). These calf thymus helicases also bind to Bio-Rex 70 column like HDH III but elute at 0.15 M NaCl while HDH III elutes at 0.80 M KCl Hence, HDH III must be different from these calf thymus helicases, which, furthermore, have different molecular weights than HDH III; calf thymus helicase I consisted of two polypeptides of 170 and 200 kDa and helicase II also consisted of two polypeptides of 100 and 130 kDa (15).

HDH III can use only ATP or dATP as energy source for the unwinding activity. Similar findings were reported for HDH I (18), HDH IV (19) and calf thymus helicase I (15). However, helicase II and helicase B from calf thymus (15, 27) and mouse helicase (28), can use all NTP's, the 47 kDa helicase from calf thymus also uses CTP and dCTP (29) and the SSB requiring DNA helicase from HeLa cells also uses CTP, dCTP and UTP (20). $Mg^{2+}$ is essential for the activity of HDH III and $Mn^{2+}$ can also replace it as reported for HDH I (18), HDH IV (19), calf thymus

Table III. Summary of Properties of HDH I, III and IV

| Property | HDH I | HDH III | HDH IV |
|---|---|---|---|
| Size kDa (by SDS PAGE) | 65 | 46 | 100 |
| Direction of unwinding | 3′ to 5′ | 3′ to 5′ | 5′ to 3′ |
| DNA-RNA unwinding[a] | ND[b] | − | + |
| RNA-DNA unwinding[a] | + | ND[b] | + |
| Blunt end duplex unwinding | − | − | − |
| Preference of fork-like structure of substrate | − | + | − |
| Requirement of free ssDNA on substrate, (bases) | > 11 | > 84 | > 84 |
| Maximum length unwound (bp) | > 40 | > 40 | < 40 |
| Cation requirements | $Mg^{2+} > Mn^{2+} > Ca^{2+}$ | $Mg^{2+} > Mn^{2+}$ | $Mg^{2+} = Mn^{2+} = Zn^{2+}$ |
| Inhibition by | | | |
| KCl or NaCl (200 mM) | − | + | + |
| $KPO_4$ (100 mM) | ± | ± | − |
| Ammonium Sulfate (45 mM) | ± | ± | − |
| ssDNA (30 μM as P) | + | + | + |
| RF DNA (30 μM as P) | − | − | ± |
| RNA (30 μM as P) | ND[b] | − | ± |

[a] DNA-RNA is a short RNA stretch annealed to a long DNA strand, RNA-DNA is the opposite
[b] Not Determined

(15, 29) and mouse (28). HDH III can not use $Ca^{2+}$ and $Zn^{2+}$ while HDH I uses also $Ca^{2+}$ and HDH IV uses $Zn^{2+}$ (Table 3). HDH III activity is inhibited by 200 mM KCl or NaCl similar to that of HDH IV (19), calf thymus helicase II (15) and calf thymus 47 kDa enzyme (29) while HDH I is not inhibited (18).

Human DNA helicase III moves apparently in a 3′ to 5′ direction along the bound strand, like our previuosly described HDH I (18), SV40 T-antigen (30), Polyoma T antigen (31), 47 kDa calf thymus DNA helicase (29), nuclear DNA helicases I and II from calf thymus (15) and HSSB-dependent DNA helicase from HeLa cells (20). Our previously described HDH IV moves instead in the 5′ to 3′ direction (19).

The most distinctive feature of HDH III is its requirement for a fork-like structure which, though not being absolute (the tailless substrate works at approximately one fourth of the rate of the tailed one) clearly distinguishes it from the other two known human helicases (18, 19) and from the described calf thymus enzymes (15). A recently purified calf thymus δ helicase (32) also shows preference for fork-like structures, but is on the other hand certainly different from our enzyme both for its size (58 kDa) and for the polarity, which is opposite to that of HDH III. In our case the preference for a partially unwound extension on the annealed sequence of the substrate is quite marked: if one looks at the summary of the results reported in Figure 6, one can see how, surprisingly, it appears irrelevant even whether the hanging tail is on the 5′ or on the 3′ of the short annealed portion. This could be interpreted as indicating that the ideal substrate for the attachment of helicase is a fork-like situation; one must infer, by considering these results together with those on the polarity reported in Figure 7, that, after the attachment to the branched substrate, the enzyme can enter the duplex area and unwind it with a satisfactory efficiency: as shown in Figure 6D, a duplex as long as 40 bp can be totally unwound by the enzyme, even though at a reduced rate. On the contrary, on an unbranched substrate, one must infer that the poor capacity of the enzyme to bind to the substrate also hampers its ability to progress along it for a prolonged extent: as one can see by comparing Figure 6E and F, the extension of the duplex area from 17 to 25 bp in a non fork-like substrate, totally inhibits the activity of the enzyme.

The results reported in Figure 6H and I, indicate also that, when confronted with an unbranched substrate, the enzyme requires a very long stretch of single stranded DNA to bind in an effective way: protruding stretches as long as 84 nucleotides appear to be insufficient to allow for the enzyme activity, which has to be compared instead to the effectiveness of a 15 nucleotide long single strand branch (Figure 6C) which allows the expression of the activity of the enzyme. It appears unlikely that an 84 nucleotide stretch (which has a length as long as 600Å) has not enough room to accomodate a 46 kDa protein, i.e. a molecule that would have a 40Å diameter in a globular shape. Thus, taking all our data into account, it seems reasonable to assume that a branched structure is required for an effective binding of the enzyme to the substrate. The alternative possibility, namely that several molecules of the enzyme have to bind to the single strand and act in a cooperative fashion [as for *E.coli* helicase (5) and probably HDH IV (19)], is ruled out by the result of Figure 6C, from which it appears that a stretch as short as 15 nucleotides can be bound, provided that it is part of a fork-like structure.

This preference for fork-like structure is certainly the most distinctive property of HDH III, but it is not sufficient to give an indication on its possible function. In fact, this preference could be consistent both with an involvement in the advancement of the growing fork, somewhat like the T antigen is doing, or with the ability to bind to areas of DNA partially unwound because of mismatches or chemical damage on one strand: the enzyme could thus be involved only or also in repair mechanisms. It seems instead unlikely that it is involved in RNA metabolism, considering (again a feature which distiguishes it clearly from the other two human helicases) that it does not unwind DNA-RNA hybrids (see Figure 8).

In order to obtain direct indications of the role of any of the described helicases in human DNA metabolism, it will be essential to utilize studies of physiological nature, both by isolating and studying the expression of the genes which code for them, and by determining the effect of in vivo inactivation of the function of each of the described helicases.

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