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Human DNA helicase II: a novel DNA unwinding enzyme identified as the Ku autoantigen

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Human DNA helicase II (HDH II) is a novel ATP-dependent DNA unwinding enzyme, purified to apparent homogeneity from HeLa cells, which (i) unwinds exclusively DNA duplexes, (ii) prefers partially unwound substrates and (iii) proceeds in the 3′ to 5′ direction on the bound strand. HDH II is a heterodimer of 72 and 87 kDa polypeptides. It shows single-stranded DNA-dependent ATPase activity, as well as double-stranded DNA binding capacity. All these activities comigrate in gel filtration and glycerol gradients, giving a sedimentation coefficient of 7.4S and a Stokes radius of ~46 Å, corresponding to a native molecular weight of 158 kDa. The antibodies raised in rabbit against either polypeptide can remove from the solution all the activities of HDH II. Photoaffinity labelling with [α-^{32}P]ATP labelled both polypeptides. Microsequencing of the separate polypeptides of HDH II and cross-reaction with specific antibodies showed that this enzyme is identical to Ku, an autoantigen recognized by the sera of scleroderma and lupus erythematosus patients, which binds specifically to duplex DNA ends and is a regulator of a DNA-dependent protein kinase. Recombinant HDH II/Ku protein expressed in and purified from *Escherichia coli* cells showed DNA binding and helicase activities indistinguishable from those of the isolated protein. The exclusively nuclear location of HDH II/Ku antigen, its highly specific affinity for double-stranded DNA, its abundance and its newly demonstrated ability to unwind exclusively DNA duplexes, point to an additional, if still unclear, role for this molecule in DNA metabolism.

Key words: DNA binding protein/DNA-dependent ATPase/DNA helicase/Ku autoantigen/unwinding enzyme

Introduction

The unwinding of parental DNA strands during replication, repair, recombination and, in some cases, transcription, is catalysed by DNA helicases. The energy requirement for this reaction is provided by the hydrolysis of nucleoside or deoxynucleoside 5′ triphosphates; hence all the helicases possess intrinsic DNA-dependent ATPase activity (Geider and Hoffmann-Berling, 1981; Kornberg and Baker, 1991). A large number of DNA helicases have been identified in bacteriophage, bacterial, viral and eukaryotic systems (Matson and Kaiser-Rogers, 1990; Lohman, 1992, 1993; Thommes and Hübscher, 1992).

Previously we have demonstrated the presence in HeLa cells of five different human DNA helicases (HDH I–V). We have described the purification to homogeneity and properties of HDH I (Tuteja *et al.*, 1990b), HDH III (Tuteja *et al.*, 1992), HDH IV (Tuteja *et al.*, 1991) and HDH V (Tuteja *et al.*, 1993); these appear different from other helicases isolated from the same source, namely helicase ε (Seo *et al.*, 1991), having a strict requirement for homologous single-stranded (ss) DNA binding protein, and helicase α, for its size (Seo and Hurwitz, 1993). In the course of our previous work, we observed that the chromatographic fractions containing HDH II activity also contained a DNA binding activity reminiscent of the Ku antigen. This is an abundant nuclear protein, first identified as an autoantigen, recognized by scleroderma and lupus erythematosus sera. Recently, Ku was shown to be a regulator of a DNA-dependent protein kinase (Lees-Miller *et al.*, 1990; Dvir *et al.*, 1993; Gottlieb and Jackson, 1993; Woodgett, 1993). Ku binds to the termini of duplex DNA, regardless of their chemical features and sequences, and then slides along the termini in several copies like beads on a string, with a 25 bp periodicity and without an energy requirement (Mimori and Hardin, 1986; May *et al.*, 1991; Blier *et al.*, 1993; Csordàs-Toth *et al.*, 1993; Woodgett, 1993).

Because of these unusual properties, Ku has often misled investigators by behaving, at first sight, like a sequence-specific DNA binding protein. In fact, in a bandshift assay Ku becomes 'trapped' by the DNA molecule (by binding to ends and then sliding along the duplex) and is no longer available for binding other DNA competitors added subsequently, thus mimicking the behaviour of a sequence-specific DNA binder. In our laboratory, in the course of a study on the proteins interacting with a human DNA region containing a replication origin, we also obtained initial evidence for apparent specific binding by an unknown protein to a 25 bp probe; complete purification of this DNA binding protein by gel retardation assay led to the purification of Ku, identified by its size and N-terminal sequence (Csordàs-Toth *et al.*, 1993). Appropriate competition experiments (in which the cold competitor was added before Ku) and *in vitro* footprinting again demonstrated the affinity for DNA duplex ends (without apparent sequence specificity) typical of this molecule (Csordàs-Toth *et al.*, 1993). The pure Ku, so isolated, contained a significant and unambiguous helicase activity; independent purification of this activity,

Table I. Purification of HDH II

Fraction	Step	Volume (ml)	Protein (mg)	DNA helicase activity		DNA binding activity	
				total (U)	specific (U/mg)	total (U)	specific (U/mg)
	nuclear extract	495	1881	ND		ND	
I	NH_4SO_4 precipitate	230	943	ND		886	0.94
II	Bio-Rex 70	130	178	ND		350	1.97
III	FPLC-MonoQ	16	7.2	46 170	6412	136	18.8
IV	dsDNA–Sepharose	2.8	0.26	17 100	65 769	107	412

ND = not determined.

as described below, demonstrated that the two properties resided in the same molecular species. This was further confirmed by the properties of the recombinant proteins produced in *Escherichia coli* following appropriate expression cloning.

In this report we describe the purification and characterization of HDH II and we show that it is, in fact, identical to the Ku antigen.

Results

Purification of HDH II

The DNA helicase and DNA binding activities were measured in all the purification steps as described in Materials and methods. The substrate used for the helicase assay is an M13 molecule to which a 17 nucleotide fragment is annealed, having hanging 5′ and 3′ tails (see Tuteja *et al.*, 1992; Figure 8A); the 25 bp blunt end duplex DNA probe used for electrophoretic mobility shift assay was the same as that reported by Csordàs-Toth *et al.* (1993), and shown in Figure 8Z. The results of the purification of the DNA helicase and DNA binding activities are shown in Table I. The enzyme was purified from 330 g of frozen HeLa cell pellet; all the steps were carried out at 0–4°C. Nuclear extract preparation, ammonium sulfate fractionation and Bio-Rex 70 column chromatography using buffer A were performed as described previously (Tuteja *et al.*, 1990b, 1992). The fractions containing both activities eluted at ~0.3 M NaCl in buffer A (fraction II, 130 ml). Fraction II was dialysed in buffer B and applied on an 8 ml FPLC MonoQ column; the fractions which eluted at ~0.24 M NaCl containing both activities were purified further (fraction III, 16 ml). Fraction III was dialysed in buffer C and affinity chromatographed on a double-stranded (ds) DNA–Sepharose column. The fractions containing part of the helicase activity and all of the binding capacity eluted at 0.3 M NaCl (fraction IV, 2.8 ml). Fraction III also contained another helicase activity that was removed in the last step and will be characterized in the future. Since the early fractions of the purification contained several other helicases, which separated in different fractions, and also contained interfering activities (DNases), no quantitative determination of helicase activity could be performed until fraction III. Also, in the last step the removal of the different helicases mentioned above caused a variation in the helicase:DNA binding capacity ratio. Fraction IV had the same ratio of helicase to binding capacity as an independent preparation of Ku (Csordàs-Toth *et al.*, 1993), was similarly free of DNA topoisomerase, ligase, polymerase, nicking and hydrolysing activities, and similarly contained only two polypeptides of 72 and 87 kDa, present in approximately equal amounts on SDS–PAGE (Figure 1A). These M_r values correspond to those reported for the Ku heterodimer (Mimori and Hardin, 1986). No contaminant protein was evident: if present, it should be <2% of the silver-stainable material.

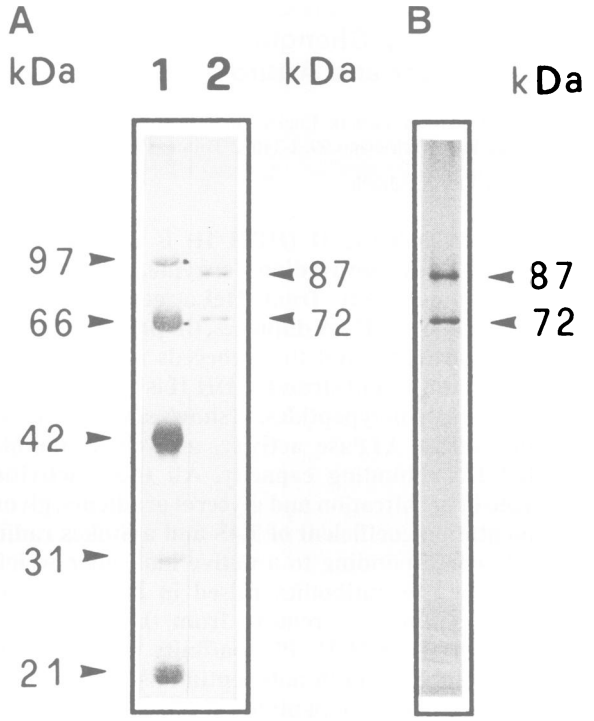

Fig. 1. (**A**) SDS–PAGE of HDH II/Ku fraction IV (0.5 μg lane 2) and M_r markers (lane 1). (**B**) Autoradiogram of photoaffinity labelled HDH II/Ku (fraction IV, 0.2 μg) with [α-^{32}P]ATP. The labelling was performed as described previously (Tuteja *et al.*, 1993).

ssDNA-dependent ATPase activity was present at a level of ~93 pmol of ATP hydrolysed for 30 min by 90 ng of pure HDH II. The UV-mediated covalent labelling of the pure preparation with [α-^{32}P]ATP showed labelling of both the 72 and 87 kDa polypeptides (Figure 1B), indicating that ATP must come into close contact with both subunits.

In view of this observation and the fact that Ku has been demonstrated recently to be a cofactor as well as a substrate of a DNA-dependent protein kinase of 350 kDa (Lees-Miller *et al.*, 1990; Dvir *et al.*, 1992, 1993; Gottlieb and Jackson, 1993), we assayed the purified material for the possible presence of self-phosphorylating activity. No

Table II. Reaction requirements of the purified HDH II/Ku activity

Reaction conditions	Percentage unwinding[a]
Complete	82
− Enzyme	<2
− ATP	<2
− ATP + dATP (4 mM)	80
− ATP + ATPγS (4 mM)	<2
− ATP + ADP (4 mM) or AMP (4 mM)	<2
− ATP + CTP (4 mM) or dCTP (4 mM)	10
− ATP + GTP (4 mM) or dGTP (4 mM)	5
− ATP + dTTP (4 mM)	5
− ATP + UTP (4 mM)	10
− $MgCl_2$	<2
− $MgCl_2$ + $CaCl_2$ (1 mM)	<2
− $MgCl_2$ + $ZnSO_4$ (1 mM)	<2
− $MgCl_2$ + $MnCl_2$ (1 mM)	90
− $MgCl_2$ + $CdCl_2$ (1 mM)	<2
− $MgCl_2$ + $CuCl_2$ (1 mM)	<2
− $MgCl_2$ + $NiCl_2$ (1 mM)	<2
− $MgCl_2$ + $AgNO_3$ (1 mM)	<2
− $MgCl_2$ + $CoCl_2$ (1 mM)	<2
+ KCl or NaCl (200 mM)	6
+ EDTA (5 mM)	<2
+ M13 ssDNA (30 μM as P)	<2
+ M13 RFI DNA (30 μM as P)	81
+ *E.coli* t-RNA (30 μM as P)	85
+ Trypsin (1 U)	<2
+ Poly(A), (C) or (U) (30 μM as P)	80
+ Poly(G) (30 μM as P)	10
+ Histone H1 (1 μg/ml)	<2

[a]Helicase reaction was carried out with 90 ng of pure protein (fraction IV) with 1 ng of the substrate (Figure 8A). P, phosphate.

such activity was detected in this preparation, in either the presence or absence of DNA, in agreement with the lack of a 350 kDa protein in the Ku preparation (see Figure 1); also, the purified HDH II is not a substrate for the cdc2 and CKII protein kinases.

Characterization of HDH II

The main properties of HDH II as a helicase are reported in Table II. The pure enzyme is heat labile and loses both helicase and DNA binding activities upon heating at 56°C for 5 min. The helicase activity was destroyed by trypsin and inhibited by 200 mM NaCl or KCl or by EDTA (5 mM), M13 ssDNA (30 μM as phosphate) and histone (1 μg/ml). The M13 replicative form I DNA and *E.coli* t-RNA at 30 μM as phosphate had no effect. The optimum concentrations of ATP, $MgCl_2$ and KCl for helicase activity are 4, 1 and 60 mM, respectively. Without ATP or $MgCl_2$ the activity was not detectable (Table II).

The kinetics of unwinding with 90 ng of HDH II shows a linear rate up to 30 min (Figure 2C) and deviates from linearity with longer incubation. Titration of helicase activity with increasing amounts of HDH II showed a maximum value of unwinding of ~90% in 30 min with 180 ng of the enzyme (Figure 2D). Saturation was reached with ~90 ng (2.6 U).

If the helicase activity was present as a contaminant, having the same size as either polypeptide observed in SDS−PAGE, one would expect that it could be separated by size fractionation in non-denaturing conditions. Hence, the native molecular weight of pure HDH II was measured by sedimentation in a glycerol gradient (Figure 2A) and gel filtration (Figure 2B), as described by Siegel and Monty (1966). DNA helicase, ssDNA-dependent ATPase and DNA binding activities migrated together and gave a molecular weight of 158 kDa, with a sedimentation coefficient of 7.4S and a Stokes radius of 46 Å, corresponding to an f/f_0 ratio of 1.3 and an axial ratio of ~6. These data are in excellent accordance with a rather elongated structure of a heterodimer of the 72 and 87 kDa subunits, as demonstrated to be the case for the Ku antigen (Mimori and Hardin, 1986).

Microsequencing of the isolated 87 kDa band (Figure 3A) gave the N-terminal sequence of the Ku large subunit (Yaneva *et al.*, 1989). The 72 kDa subunit did not give an N-terminal signal, which is in accordance with the fact that this subunit is acetylated (Reeves and Sthoeger, 1989). After acid cleavage of the 72 kDa subunit, one of the resulting fragments gave a correct internal sequence of the Ku antigen small subunit (Figure 3B).

Antibody neutralization and Western blotting

Purified HDH II was individually reacted with immunoglobulin (Ig) G purified from pre-immune rabbit sera and from sera raised against either the 72 or 87 kDa subunit, as well as with a mixture of the two immune IgG; the complex was removed by protein A−Sepharose. When the supernatant was analysed for helicase and DNA binding activities, it was observed that both activities were removed, regardless of the antibody used, whereas there was no activity reduction in the pre-immune IgG-treated samples (Figure 4A and B).

The Western blotting results showed that anti-87 and anti-72 subunit antibodies recognized exclusively the respective antigens in the crude nuclear extract as well as in the pure HDH II fractions (Figure 5A and B). The 87 kDa polypeptide was also recognized in nuclear extract as well as in the pure HDH II fraction by an antibody against the large subunit of Ku (Figure 5C; kindly provided by Dr David Lane). These results also demonstrate that the 72 kDa subunit is not a degradation product of the larger one.

Cloning, expression and reconstitution of the HDH II/Ku heterodimer

The 72 and 87 kDa subunits of the Ku antigen were expressed in a T7 promoter-based expression system. Originally the pRSET expression vectors (Schoepher, 1993) were used (kindly donated by R.Schoepher) in BL21(DE3)[pLysS] or [pLysE] *E.coli* strains. Small-scale experiments showed a high level of expression for both subunits (~10 and 20% of the total cellular protein for the 87 and 72 kDa subunits, respectively). Such high expression levels, however, could be achieved on a larger scale only after subcloning into the lower copy number pET11a vector (Studier *et al.*, 1990), which contains lac operator-controlled T7 promoter (T7lac). The final expression constructs, pET6bKu72 and pET5aKu87, produced fusion proteins with short N-terminal extensions (27 and 24 amino acids, respectively). The fusion partners contain the first 11 amino acids of the T7 phage gene 10, a hexahistidine, the factor Xa cleavage site, as well as a few amino acids derived from linker sequences used in the subcloning steps. Expression of the fusion proteins is shown in Figure 6 (lanes 3 and 5). Western blotting,

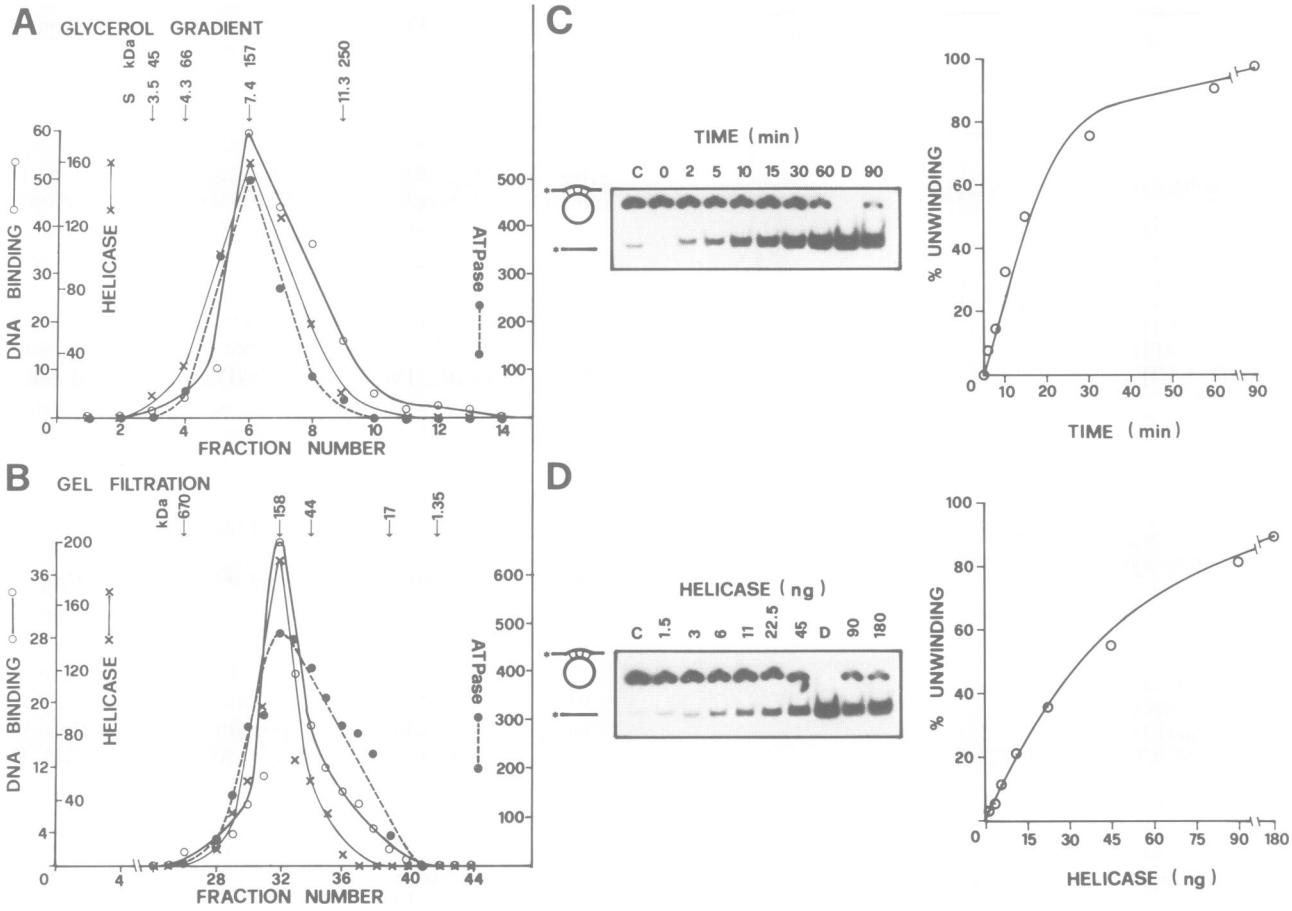

Fig. 2. (**A**) Glycerol gradient (15–35%) centrifugation of 20 μg of concentrated fraction IV at 55 000 r.p.m. for 16 h at 4°C in an SW 65 rotor. The distribution of DNA helicase, DNA binding and ssDNA-dependent ATPase activities and the positions of M_r markers are shown. (**B**) Gel filtration of 30 μg of concentrated fraction IV on a Bio-Rad SEC 400-5 column. Time (**C**) and concentration (**D**) dependence of HDH II/Ku unwinding reaction. The graphs report the quantitation of the autoradiogram data. Lane C is the control (without enzyme) and lane D is the heat-denatured substrate.

using subunit-specific Ku antibodies, showed specific immunoreaction with the corresponding recombinant subunit without cross-reaction with the other subunit or any *E.coli* protein (results not shown).

The recombinant proteins accumulated in inclusion bodies which were isolated by standard methods (Nagai and Thøgersen, 1987). Further purification by metal affinity chromatography under denaturing conditions (8 M urea or 6 M guanidium hydrochloride solutions) was unsuccessful. Gel filtration was therefore performed in 6 M guanidium hydrochloride, resulting in ~90% pure fractions for both subunits. Refolding by dialysis resulted in a soluble 87 kDa subunit, whereas the 72 kDa subunit showed a strong tendency to precipitate during dialysis. However, the refolding of a mixture of the two subunits in equimolar amounts (400 μg total protein in 1 ml) resulted in a clear solution, ~90% pure, containing the two subunits (Figure 6, lane 6). Removal of the N-terminal extensions by using factor Xa was unsuccessful; only a small amount of specific cleavage was observed at low enzyme concentrations, while higher concentrations of enzyme caused non-specific degradation of both subunits. Therefore, the recombinant Ku antigen used in further assays contained the short N-terminal extensions (see Materials and methods).

Electrophoretic mobility shift and helicase assays were performed in parallel with purified HeLa HDH/Ku antigen

A

```
1                    20
MVRSGNKAAVVLCMDVGFTM - Ku
   | ||| ||| | ||| |
XXXGXKAAVVLXMXVGFXM - HDH II
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B

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343          351
PGLMLMGFKP - Ku
   ||||||||
XXLMLMGFKP - HDH II
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Fig. 3. Microsequencing of the subunits of HDH II/Ku antigen. The 87 kDa band gave an N-terminal sequence identical to that of the Ku large subunit (**A**). The 72 kDa subunit did not give an N-terminal signal, probably due to acetylation. Sequencing of an N-terminus liberated by mild acid cleavage gave the correct sequence of the Ku small subunit corresponding to the residues vicinal to the only acid scissile bond in that protein (**B**). X = residues not properly identified by the sequencing procedure.

and with the recombinant Ku antigen on the refolding product. These assays showed that the same activities were present in both preparations, although the recombinant protein showed ~5% specific activity in DNA binding

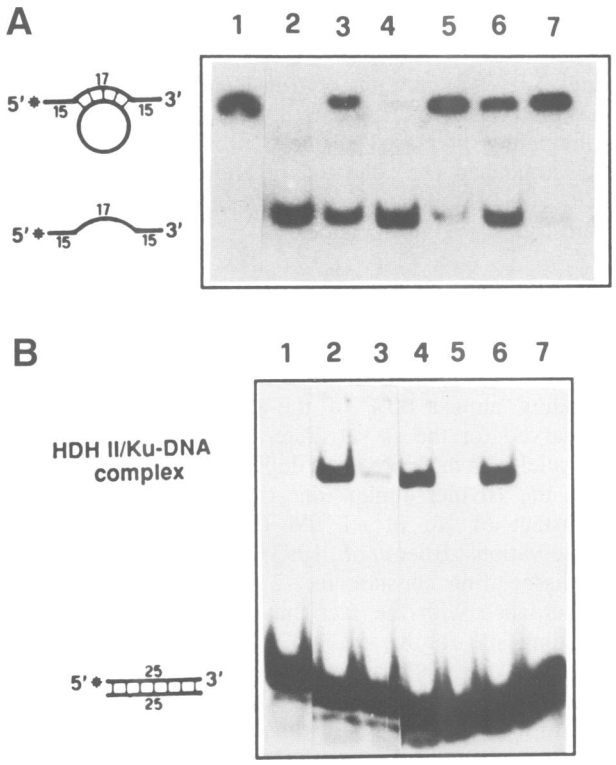


Fig. 4. Immunodepletion of DNA helicase activity (**A**) and DNA binding activity (**B**) of HDH II/Ku. In both panels lane 1 is control without enzyme; lanes 2, 4 and 6, HDH II pretreated with IgG of pre-immune sera; lanes 3, 5 and 7, HDH II pretreated with IgG of anti-72, anti-87 and anti-72 plus anti-87, as described in Materials and methods.

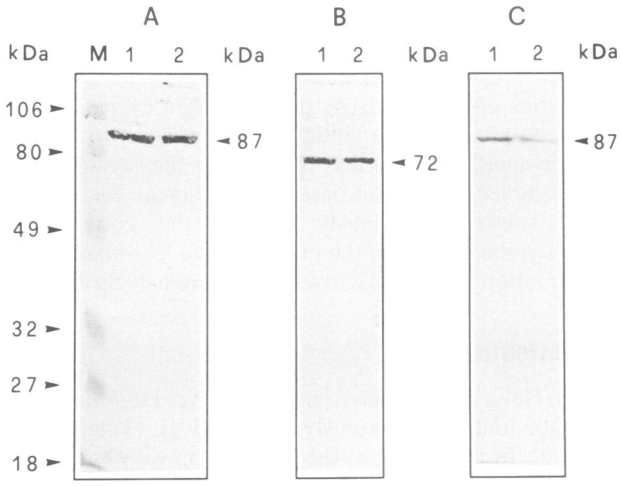


Fig. 5. Western blotting with anti-87 (**A**), anti-72 (**B**) and anti-Ku against large subunit (**C**) antibodies. In each panel lane 1 contained 20 μg of nuclear extract (fraction 1) and lane 2 contained 0.5 μg of HDH II (fraction IV). Lane M, molecular weight markers.

assay and ~40% specific activity in helicase assay as compared with the HeLa protein (see Table III). These quantitative differences could be attributed to the presence of the short N-terminal extensions in the recombinant subunits or, possibly, to the lack of post-translational modifications which might be necessary for full activity. To remove any trace of *E.coli*-derived protein and to assess whether the reconstituted molecule had the same size as the natural heterodimer, we subjected 400 μg of

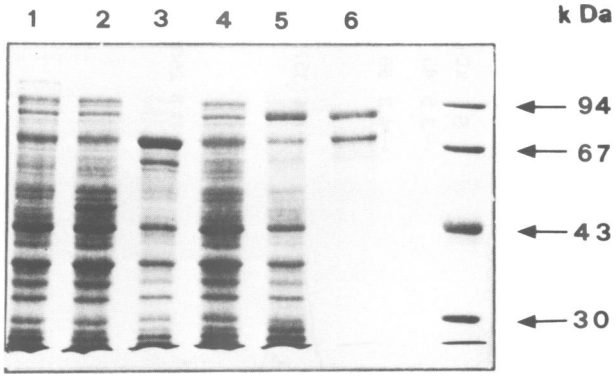


Fig. 6. Expression of Ku subunits in BL21(DE3)[pLysS] *E.coli*. Total cell extracts corresponding to 50 μl uninduced or IPTG-induced cultures were analysed on SDS−10% polyacrylamide gel. Lane 1, induced host cell without expression vector; lane 2, pET6bKu72 uninduced; lane 3, pET6bKu72 induced; lane 4, pET5aKu87 uninduced; lane 5, pET5aKu87 induced; lane 6, refolded recombinant Ku protein. Molecular weight standards (Pharmacia) are indicated on the right-hand margin. Proteins are visualized by Coomassie brilliant blue staining.

Table III. Comparison of specific activities of HeLa-derived and recombinant forms of HDH II/Ku

Fraction	DNA helicase activity (%)	DNA binding activity (%)
HeLa-derived HDH II/Ku	100	100
Reconstituted recombinant HDH II/Ku		
Refolded protein	40	5
dsDNA−Sepharose	65	15
Glycerol gradient fraction 6	70	20

the refolded product to the same dsDNA chromatography as the HeLa-derived proteins. The recombinant proteins behaved on the column as the natural ones (fraction III, see above); the chromatographed product (containing equimolar amounts of the two subunits: see Figure 7, lane A) was analysed for its sedimentation coefficient in a glycerol gradient (as for fraction IV in the experiment reported in Figure 2A); the DNA binding and helicase activities migrated with an sedimentation coefficient of 7.4S, like the natural molecule, showing that we had reconstituted a heterodimer of the two subunits (as shown also by the SDS−PAGE analysis of the peak fractions; see Figure 7, lanes C−E). The specific activities of the chromatographed and size-fractionated forms gave values higher than those of the refolding reaction products for both types of activity (Table III).

On the basis of the co-purification, the native and subunit M_r, the sequence analysis, the immunological identity and the properties of the molecules reconstituted from the recombinant proteins, we conclude that the Ku antigen is also a helicase, namely, HDH II. HDH II/Ku lacks in its sequence the standard structural motifs reported for some DNA helicases (Gorbalenya *et al.*, 1989). This is not surprising in itself, since these motifs rely on purely empirical observation and are not apparently justified on functional grounds, so much so that they can also be present in proteins devoid of helicase activity (Selby and Sancar, 1993).

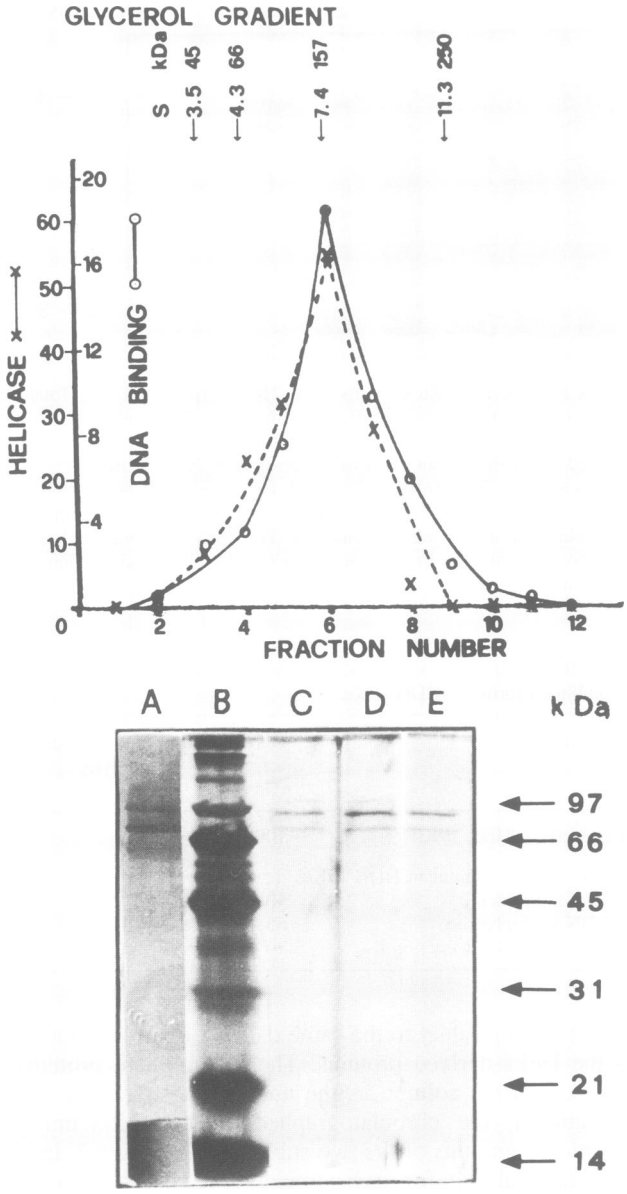


Fig. 7. Glycerol gradient centrifugations of 40 μg of the refolded and dsDNA chromatography fractionated recombinant proteins. The gradient conditions are as in Figure 2A. The distribution of DNA helicase and DNA binding activities and the positions of M_r markers are shown in the upper panel. The lower panel shows the SDS–PAGE analysis of the dsDNA–Sepharose-purified protein (lane A), of the glycerol gradient fractions 5 (lane C), 6 (lane D) and 7 (lane E), and of the M_r markers (lane B). Proteins are visualized by silver staining.

DNA helicase activity with various substrates

The helicase activity, using ~1 ng of different types of substrate with 90 ng of HDH II, is shown in Figure 8A–R. The results show that HDH II prefers a fork-like substrate for maximum unwinding (Figure 8A–C) as compared with a substrate without a tail (Figure 8D). The substrate with a 5′-tailed fork structure is preferred to the 3′-tailed one. HDH II still shows a 7% unwinding capacity if the hybrid region of the tail-less substrate increases to 32 bp (Figure 8E), but it does not show unwinding with a duplex region of 49 bp even if it has tails on both ends (Figure 8F). HDH II cannot unwind blunt-ended duplexes (Figure 8G). There was no unwinding with small linear ds substrates with nicks (Figure 8I) or with a ssDNA portion of 84 nucleotides or less (Figure 8H and J–M), even if they contained a 5′ or 3′ end tail (Figure 8J and K). The direction of translocation of HDH II was exclusively 3′ to 5′ (Figure 8N and O). The efficiency of unwinding increased if the 3′ to 5′ direction-specific substrate had a 5′ end tail of eight nucleotides (Figure 8P). HDH II failed to unwind DNA–RNA or RNA–RNA substrates (Figure 8Q and R).

DNA binding activity

The binding affinity of HDH II/Ku for linear duplexes is much greater than for its ss counterpart. In fact, as Figure 8S–Z* shows, at an HDH II/Ku concentration capable of binding almost 60% of the duplex, <5% binding is observed for the ss substrate. The binding increases in parallel with the increase of duplex regions in the substrate. For the 101mer duplex, one can estimate a dissociation constant of Ku of ~1 nM (in agreement with other observations; Blier *et al.*, 1993) and for the 25mer duplex, a dissociation constant of ~2 nM. The difference is in accordance with the fact that the 25mer has only one binding site for Ku (each Ku molecule covers ~25 bp; Blier *et al.*, 1993), whereas the longer ligand has two ends available for binding. The affinity for ssDNA is much lower and can be estimated to be ~0.3 μM for the 101mer (data not shown). The fork-like substrate used for the helicase assay gives half-maximal velocity (when presumably 50% of the substrate is bound to the enzyme) at 19 nM concentration of HDH II/Ku. Qualitatively speaking, the affinity of HDH II/Ku for the helicase optimal substrate (Figure 8A) lies somewhere between its duplex and ssDNA binding affinities.

Substrate inhibition studies

Figure 9 reports the effect on the helicase or DNA binding activities of different substrates added in excess to either reaction mixture. One could expect that the two activities might interfere with each other since they reside on the same molecule. As the data show, whereas each specific cold substrate obviously inhibits the corresponding activity, the effects on the other one are less marked. This observation will be discussed more extensively below.

Discussion

From HeLa cells we have purified a novel DNA unwinding enzyme defined previously as HDH II (Tuteja *et al.*, 1990b). In the course of the purification we observed that HDH II fractions also contained a DNA binding activity indistinguishable from that of Ku antigen and that, throughout the whole fractionation process, DNA helicase and DNA binding activities co-purified. From the parallel behaviour of the two activities during the purification procedure, their identical migration properties in sedimentation and gel filtration, the size of the isolated subunits of HDH II, the protein sequencing data, the immunological data and the properties of the recombinant proteins expressed in *E.coli*, we conclude that Ku antigen and HDH II are the same molecule: namely, a heterodimer of 72 and 87 kDa subunits.

The properties of HDH II/Ku as a helicase distinguish it clearly not only from the other four DNA helicases described by our group, as mentioned, but also from the

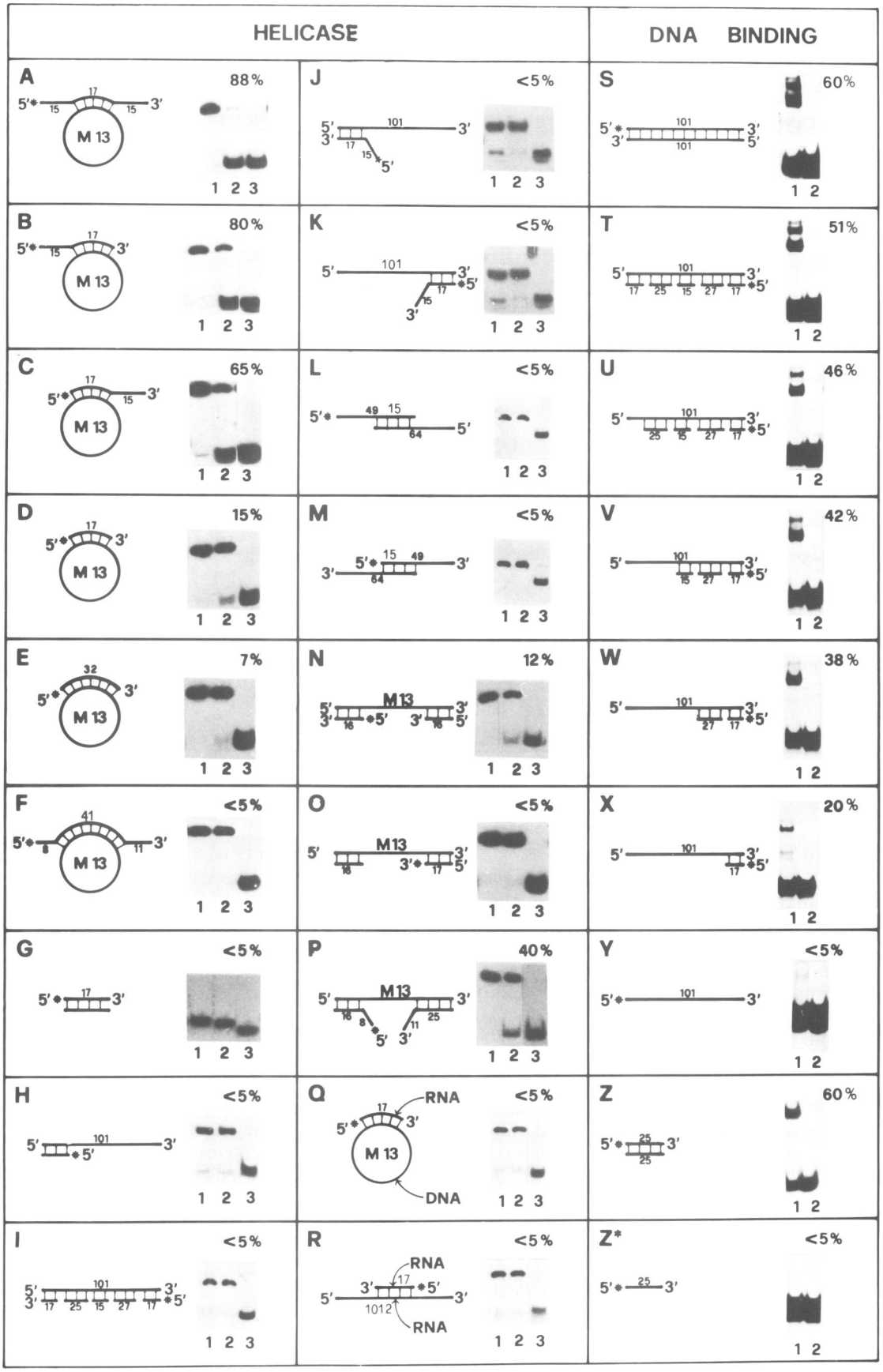


Fig. 8. DNA helicase and DNA binding activities with different substrates. For helicase, 90 ng of fraction IV and 1 ng of substrate, whereas for DNA binding, 4 ng of fraction IV and 0.2 ng of substrate, were used. The asterisks denote the ^{32}P-labelled end. Each panel shows the structure of the substrate used, an autoradiogram of gel and the percentage unwinding (**A**−**R**) or percentage binding (**S**−**Z***). From (A) to (R), lane 1 is control without enzyme, lane 2 is reaction with enzyme and lane 3 is heat-denatured substrate. From (S) to (Z*), lane 1 is reaction with enzyme and lane 2 is control without enzyme.

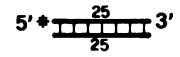
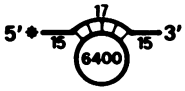
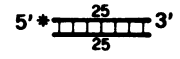
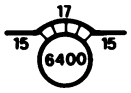
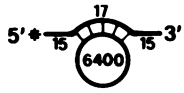
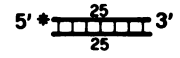
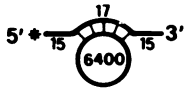
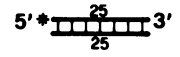
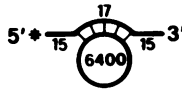
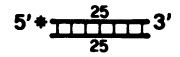
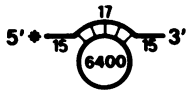
HDH II/Ku - 62.5 nM BINDING CAPACITY			COMPETITORS FOR DNA BINDING AND HELICASE ACTIVITY		HDH II/Ku - 62.5 nM HELICASE ACTIVITY		
SUBSTRATE STRUCTURE	CONCENTRATION	INHIBITION (%)	STRUCTURE	CONCENTRATION	SUBSTRATE STRUCTURE	CONCENTRATION	INHIBITION (%)
	1.2 nM	87		102 nM		0.047 nM	100
	1.2 nM	88		102 nM		0.047 nM	100
	1.2 nM	0		19 nM		0.047 nM	0
	1.2 nM	80		19 nM		0.047 nM	0
	1.2 nM	100 100 100 100 100		4.2 µM 8.4 µM 13 µM 17 µM 21 µM		0.047 nM	14 42 54 63 77

Fig. 9. Competition of DNA binding and helicase activities of HDH II/Ku with different DNA substrates. The conditions and the controls of the respective assays are reported in Figure 8A and Z.

two helicases (α and ε) purified from the same source by Seo *et al.* (1991) and Seo and Hurwitz (1993), since the former has a different size and the latter has an absolute requirement for ssDNA binding protein.

As indicated above, for maximum activity HDH II prefers a fork-like structure, as reported previously for HDH III (Tuteja *et al.*, 1992) and δ helicase from calf thymus (Li *et al.*, 1992). Unlike HDH III, HDH II works somewhat more efficiently if the substrate has a 5′ end tail as compared with a 3′ end tail, suggesting that HDH II translocates in the 3′ to 5′ direction. By using the direction-specific substrate we confirmed that it moves in a 3′ to 5′ direction along the bound strand, like the previously described HDH I (Tuteja *et al.*, 1990b), HDH III (Tuteja *et al.*, 1992), HDH V (Tuteja *et al.*, 1993), DNA helicase α and DNA helicase ε from HeLa cells (Seo *et al.*, 1991; Seo and Hurwitz, 1993), 47 kDa calf thymus DNA helicase (Thommes and Hübscher, 1990), calf thymus DNA helicase E (Turchi *et al.*, 1992) and I (Zhang and Grosse, 1991), SV40 large-T antigen (Stahl and Knippers, 1987) and polyoma T-antigen (Seki *et al.*, 1987).

One distinguishing property of HDH II as compared with HDH I, III, IV and V is that it can utilize, to a small extent, all the other NTPs or dNTPs, and not only ATP or dATP, as cofactor, as observed also for DNA helicase α from HeLa cells (Seo and Hurwitz, 1993), helicase II and helicase B from calf thymus (Zhang and Grosse, 1991; Thommes *et al.*, 1992), and mouse helicase (Seki *et al.*, 1987).

As the Ku molecule is known to be part of a larger protein complex, the question arises as to whether the helicase activity might be due to a protein component that is tightly associated with the Ku molecule. We can rule out this possibility for the following reasons.

(i) The helicase activity belongs to a molecule whose native M_r is identical with that of Ku. A helicase molecule bound to Ku would cause the helicase activity to appear at an M_r higher than this value (or at least different, if it dissociates from Ku during size fractionation; Figure 2). (ii) Silver staining and sequencing did not show any contaminating proteins (Figures 1 and 3): components down to 2% of the total protein would have been detectable by silver staining. (iii) The antibodies raised against the isolated subunits can remove both the helicase and the DNA binding activities (Figure 4). (iv) The known helicases detected in HeLa cells all have substrate specificities that are different from HDH II. Only HDH III (Tuteja *et al.*, 1992) has a preference for fork-like structures, which is somewhat reminiscent of HDH II. However, as discussed above, HDH III differs in several other aspects (including size, 46 kDa) from HDH II and has a significantly lower specific activity than the latter, so that an HDH III contamination (which must be present in <2% of the protein content) simply cannot be the cause

of the DNA unwinding activity detected here. (v) Finally, the recombinant proteins purified from *E.coli* could reform a heterodimer with molecular and functional properties indistinguishable from those of the same molecular species purified to homogeneity from HeLa cells. Thus, we are left with the inevitable conclusion that the helicase and DNA binding activities reside on the same molecule.

Little similarity is observed between the optimal substrates of HDH II/Ku as a helicase and as a binding protein, respectively; also, duplex DNA binding is unaffected by ATP. Does the excess of either substrate inhibit the functionality of the molecule on the other one? As Figure 9 shows, this indeed happens in both directions but at very high excesses of either cold competitor, indicating that the enzyme can still act in either function when bound to the other substrate. In fact, the 25mer duplex substrate, added in 10^5-fold molar excess with respect to the helicase substrate (4 μM versus 50 pM), hardly affected the helicase rate, even though at that concentration the great majority of Ku molecules are certainly bound to the 25 bp duplex. Conversely, molecules that are not substrates for either function (covalently closed ds circles) do not affect either reaction. It is interesting to note that nicked ds circles compete in binding to the DNA but not with the helicase activity (Figure 9).

The observation that Ku antigen has ATP-dependent DNA unwinding activity suggests that it plays an important role in chromosomal transactions. In fact, this heterodimeric protein, present exclusively in the nuclei of eukaryotic cells, is quite abundant (~5×10^5 molecules/nucleus) and does not interact appreciably with RNA (Mimori and Hardin, 1986; this work; Figure 8Q and R). Also, recent observations show that Ku antigen binds to nicks in duplexes (Blier *et al.*, 1993; this work, Figure 9) and to the areas of transition between ss and duplex DNA (Falzon *et al.*, 1993; the latter was in agreement with our observation that it has a helicase activity). These properties, together with the newly demonstrated ability to unwind duplex regions even when (most probably) bound to another DNA duplex end, point to a possible role for HDH II/Ku in some form of ATP-mediated strand exchange. This role, of course, does not preclude the participation of Ku in other cellular events, for example the regulation of the newly described DNA-dependent protein kinase, which phosphorylates several important molecules of DNA metabolism, such as RNA pol II, p53 and Ku itself (Lees-Miller *et al.*, 1990; Dvir *et al.*, 1992).

In any case, one must bear in mind that Ku antigen is probably not a homogeneous protein (Griffith *et al.*, 1992); its 72 kDa subunit was shown to be produced by a gene family and thus one can hypothesize that the cell could synthesize different variants of the dimer for different purposes (Griffith *et al.*, 1992). Therefore, our studies indicate that the helicase activity is present in at least one member of this family of molecules.

Materials and methods

Cell cultures and buffers

HeLa cells were cultured in Joklin MEM supplemented with 10% fetal calf serum (FCS), 50 μg/ml gentamycin and 2 mM glutamine, and harvested as described previously (Tuteja *et al.*, 1990b). All the buffers used for purification contained 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium metabisulfite, 1 mM pepstatin, 1 μM leupeptin and 1 mM DTT. Buffer A contained 20 mM HEPES (pH 8.0), 0.1 M NaCl, 12.5 mM $MgCl_2$, 0.2 mM EDTA and 20% glycerol. Buffer B contained 20 mM Tris–HCl (pH 8.0) instead of 20 mM HEPES. Buffer C contained 0.1% NP-40 instead of 20% glycerol.

Enzymes, vectors and E.coli strains

Restriction and DNA-modifying enzymes were obtained from New England Biolabs, Boehringer Mannheim, Promega Corp. or Pharmacia. First-strand cDNA and T7 sequencing kits were purchased from Pharmacia, and Ni-NTA resin and factor Xa from Qiagen (Diagen GmbH, Germany) and Boehringer Mannheim, respectively. Standard recombinant techniques were performed as described (Sambrook *et al.*, 1989). pRSET vectors were kindly donated by Dr R.Schoepher (Schoepher, 1993), while pET vectors and BL21(DE3)[pLysS] and [pLysE] *E.coli* strains were purchased from Novagen Inc.

DNA oligonucleotides and nucleoside triphosphates

M13mp19 ssDNA and plasmid DNA were prepared as described (Sambrook *et al.*, 1989). Poly(dI-C), NTPs and ATPγS were obtained from Boehringer Mannheim. [γ-^{32}P]ATP (185 TBq/mmol), [α-^{32}P]ATP (>15 TBq/mmol) and [α-^{32}P]dCTP (~110 TBq/mmol) were purchased from the Amersham Corporation.

All oligonucleotides were synthesized chemically and purified electrophoretically. At least 26 different oligonucleotides were used to construct various DNA substrates for helicase as well as DNA probes for electrophoretic mobility shift assays. The sequences of 19 of these DNA or RNA oligonucleotides have been previously described (Tuteja *et al.*, 1990b, 1991, 1992, 1993). The sequences of seven new ones used in this study are as follows. Oligonucleotide 1, 32mer: (5′-[A]$_{15}$TGGCCGTCGTTTTACAA-3′) contained sequences from 16 to 32 complementary to 101mer (Tuteja *et al.*, 1991) at nucleotides one to 17; used for small linear substrate with a 5′ tail (Figure 8J). Oligonucleotide 2, 32mer: (5′-AAACAGCTATGACCATG[A]$_{15}$-3′) contained sequences from one to 17 complementary to 101mer (Tuteja *et al.*, 1991) at nucleotides 85–101; used for small linear substrate with a 3′ tail (Figure 8K). Oligonucleotide 3, 25mer: (5′-GATCTCGCATCACGTGACGAAGATC-3′). Oligonucleotide 4, 25mer: (5′-GATCTTCGTCACGTGATGCGAGATC-3′) contained sequences complementary to oligonucleotide 3; used for 25 bp blunt-ended duplex DNA probe (Figures 4B and 8Z). Oligonucleotide 5, 17mer: (5′-GATCTCGCATCACGTGACGAA-3′). Oligonucleotide 6, 17mer: (5′-GATCTTCGTCACGTGATGCGA-3′) contained sequences complementary to oligonucleotide 5; used for making dsDNA affinity column. Oligonucleotide 7, 17mer: RNA oligonucleotide (5′-CUGGCCCAAUAUGGCAU-3′) complementary to the middle portion of 1 kb RNA (Tuteja *et al.*, 1990b); used for constructing RNA–RNA substrate (Figure 8R).

Preparation of helicase substrates

The structures of all the substrates (DNA–DNA, DNA–RNA and RNA–RNA) used in this study are shown in Figure 8. All the DNA substrates were prepared as described previously (Tuteja *et al.*, 1990b, 1991). The DNA–RNA substrate was prepared as described earlier (Tuteja *et al.*, 1992). For RNA–RNA substrate, the plasmid Bluescript (KS^+), containing the γ-subunit of a human retinal cGMP phosphodiesterase 1 kb cDNA (Tuteja *et al.*, 1990a), was first linearized with *Spe*I and then used for the synthesis of non-radioactive RNA using T7 RNA polymerase, as described in the Stratagene instruction manual. RNA oligo 7 (17mer, 10 ng), which is complementary to the middle portion of the *in vitro*-transcribed RNA, was first labelled at the 5′ end, as described (Tuteja *et al.*, 1990b), and then annealed with 1 μg of non-radioactive RNA in a buffer containing 50 mM Tris (pH 8.0), 6 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and 1 mM ATP. The annealing mixture was heated at 65°C for 20 min, incubated at 37°C for 1 h and then left at room temperature for 1 h. The substrate was finally purified through 1 ml of Sepharose 4B column.

Preparation of DNA probes and competitors

For blunt-end duplex oligonucleotide probes, 50 ng of oligonucleotide 3 was 5′ end-labelled by T4 polynucleotide kinase (5 U) and 2.96 MBq of [γ-^{32}P]ATP (185 TBq/mmol) in a 20 μl mixture containing 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine and 0.1 mM EDTA at 37°C for 1 h. The mixture was heated at 95°C for 2 min to destroy the kinase. This labelled oligonucleotide was annealed in the same tube by adding 2 μl of 100 mM ATP, 3 μl of 2 M NaCl and 5 μl of complementary oligonucleotide 4 (50 ng). This mixture was heated at 95°C for 15 min and was then slowly cooled to room

temperature. The probes were purified by gel filtration through a 1 ml Sephadex G-50 column.

The nicked plasmid DNA was prepared by treating 1 μg of plasmid DNA with 5 ng of DNase I, as described (Sambrook *et al.*, 1989). The ss and ds M13 DNA were prepared as described (Sambrook *et al.*, 1989).

Helicase and ATPase assays

The helicase assay measures the displacement of a labelled oligonucleotide fragment from a partial duplex molecule, catalysed by HDH II. The reaction mixture (10 μl) contained 20 mM Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 4 mM ATP, 60 mM KCl, 8 mM DTT, 4% (w/v) sucrose, 80 ng/ml bovine serum albumin, 1 ng ^{32}P-labelled helicase substrate (~1000 c.p.m.) and the helicase fraction. Incubations were performed at 37°C for 30 min, unless otherwise stated. The reaction was terminated by the addition of 1.5 μl of 75 mM EDTA, 2.25% SDS, 37.5% glycerol and 0.3% bromophenol blue; products were separated by 12% non-denaturing PAGE. The gels were dried and exposed to X-ray film. The amount of substrate displaced was quantitated by excising the radioactive bands from the gel and counting. The values were normalized to the amount of substrate displaced by heat denaturation and the background of the substrate. 1 U of helicase activity is defined as the amount of enzyme that unwinds 30% of the DNA helicase substrate at 37°C in 30 min in a linear range of enzyme concentration dependence. 1 U of RNase block was included in the reaction mixture when DNA–RNA or RNA–RNA substrates were used.

The ssDNA-dependent ATPase activities were determined in the same conditions as the helicase assay by using the method described previously (Tuteja *et al.*, 1992).

Electrophoretic mobility shift assays

DNA binding was performed in a 20 μl reaction mixture consisting of 20 mM HEPES (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, 0.2 mM EDTA, 5% glycerol, 0.1–0.2 ng ^{32}P-labelled DNA probe (generally 10 000–30 000 c.p.m.) and the protein fraction (HDH II). The competitor DNA was included wherever described. The reaction was incubated for 30 min at room temperature and the products were separated on 5% non-denaturing PAGE at +4°C. The gel was dried; the retarded bands were visualized by autoradiography and quantitated by taking the radioactive counts. 1 U of binding activity is defined as the amount of protein that binds to 1 pmol of the probe.

Preparation of dsDNA affinity column

The oligos 5 and 6 contain protruding *Mbo*I sticky ends. These oligonucleotides were annealed, phosphorylated with T4 polynucleotide kinase and ligated together with T4 ligase, as per the condition described (Sambrook *et al.*, 1989). The resulting DNA fragments (0.30–1.00 kb in size) were coupled to cyanogen bromide-activated Sepharose 4B as described (Kadonaga and Tjian, 1986).

Determination of Stokes radius

The Stokes radius of HDH II was calculated as described (Siegel and Monty, 1966) by first determining the native molecular weight by gel filtration and the sedimentation coefficient by glycerol gradient centrifugation. For gel filtration, a 300×7.8 mm column (SEC 400-5; Bio-Rad) was used on FPLC (Pharmacia) using buffer A with 5% glycerol. Pure HDH II (fraction IV) was first concentrated and ~30 μg in 50 μl were injected on this column. The column was run at a flow rate of 1 ml/min. Fractions of 0.3 ml were collected and assayed for helicase, ATPase and DNA binding activities. The column was precalibrated using gel filtration molecular weight markers in the same condition. A standard curve was generated by plotting the partition coefficient, K_{av}, versus the log of molecular weights of standard proteins. The partition coefficient, K_{av}, is equal to $(V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume of the sample, V_o is the void volume and V_t is the total volume of the gel bed. The linear range of the standard curves was used to determine the apparent molecular weight of HDH II. For glycerol gradient centrifugation, 50 μl of concentrated HDH II (fraction IV) were layered on a 5 ml 15–35% glycerol gradient in buffer A and centrifuged for 16 h at 4°C at 55 000 r.p.m. in an SW 65 rotor. Standard protein markers were also run in the same conditions. Fractions of 0.3 ml were collected from the top of the tube using HSI Auto Densi-Flow IIC (Buchler Instruments, KS) and assayed for helicase, ATPase and DNA binding activities.

Preparation of antibodies and Western blotting

The antibodies were raised in rabbit against 72 and 87 kDa proteins, separately. First the proteins were separated on SDS–PAGE and the appropriate bands were excised and mixed with Freund's complete adjuvant (Sigma) for the first injection. The rabbits were boosted at least four times every 2 weeks before the final bleeding. The antibodies were purified using protein A–Sepharose, as described (Harlow and Lane, 1988). For Western blotting, the proteins were separated on SDS–PAGE and electrophoretically transferred to nitrocellulose membrane, as described in Harlow and Lane (1988). The blotted membranes were incubated using non-fat dry milk with anti-72 or anti-87 kDa protein antibody. The antigen–antibody complexes were detected using alkaline phosphatase-conjugated goat anti-rabbit IgG H+L (Bio-Rad) as a second antibody; colour development was carried out using BCIP and NBT (Bio-Rad) according to the manufacturer's instructions.

Immunodepletion of HDH II

Aliquots (20 μl) of fraction IV were incubated separately at 4°C for 16 h with a 10-fold excess of purified controls (pre-immune IgG), anti-72, anti-87 and both anti-72 plus anti-87 antibodies. Immunodepletion was performed by adding 10 μl of protein A–Sepharose (Pharmacia) equilibrated in 100 mM Tris–HCl (pH 7.5) buffer. After 4 h at 4°C the beads were removed by centrifugation and supernatants were assayed for helicase and DNA binding activities.

Microsequencing of proteins

The purified HDH II (fraction IV) was subjected to reverse phase HPLC on an Aquapore 300 (ABI, Foster City, CA) 2.1×200 mm column. The 87 kDa band was subjected to microsequencing on an ABI 471A pulsed liquid phase sequencer. The sequencing of the 72 kDa band was performed after hydrolysing the electroblotted protein with dilute HCl, pH 2.0 at 108°C for 2 h, as described (Inglis *et al.*, 1979).

Isolation and cloning of the genes for 72 and 87 kDa subunits

Total RNA was isolated from HeLa cells (Chomczynski and Sacchi, 1987) and cDNA was prepared by using a first-strand cDNA synthesis kit (Pharmacia). PCR was performed using oligonucleotide primers **TTGTCGAC**ATCGAGGGTAGGATGTCAGGGTGGGAGTCATA and **TTGTCGAC**TCAGTCCTGGAAGTGCTTGG (72 kDa subunit, *Sal*I sites in bold), and ATGGTGCGGTCGGGGAATA and CTATATCATGTCCAATAAAT (87 kDa subunit). The PCR product of the 72 kDa subunit was cleaved with *Sal*I and cloned into the pQE11 vector (Qiagen, Diagen GmbH, Germany). The *Bam*HI and *Hin*dIII flanking sites of this vector were used to obtain a *Hin*dIII (partial)–*Bam*HI fragment which was then cloned into pRSET6b (Schoepher, 1993), resulting in pREST6b72. The PCR product of the 87 kDa subunit was cloned into *Hin*cII-cleaved pUC18 (in an orientation opposite to the LacZ' region); then, using the vector-flanking sites, the *Bam*HI–*Hin*dIII fragment was isolated and cloned into pRSET5a (Schoepher, 1993). The expression constructs were transformed into the BL21(DE3)[pLysS] *E.coli* expression strain from Novagen (Studier *et al.*, 1990). On induction with IPTG the expression of both subunits could be detected, but the scale-up proved to be difficult and poorly reproducible. The genes were therefore recloned into the lower copy number pET11a vector (Novagen), in which the T7 promoter is under the control of the lac operator. During these recloning steps a hexahistidine affinity tag and a factor Xa-specific proteolytic site were also introduced, resulting in fusions coding for N-terminal extensions MASMTGGQQMGRDQAHHHHHHLDIEGR for Ku72 and MASMTGGQQMGRGSHHHHHHIEGR for Ku87, respectively. The final expression constructs pET6bKu72 and pET5aKu87 were verified by nucleotide sequencing (T7 kit from Pharmacia). The coding sequence of the Ku87 gene was identical to the published sequence (Mimori and Hardin, 1986), while that of Ku72 contained silent mutations at amino acids 89 (GGC to GGT) and 593 (GGT to GGG) as compared with the published sequence (Reeves and Sthoeger, 1989). Details of the expression constructions are available upon request.

Expression and purification of recombinant HDH II/Ku protein

BL21(DE3)[pLysS] *E.coli* cells transformed with either pET6bKu72 or pET5aKu87 were grown at 37°C in 1 l of LB medium (Sambrook *et al.*, 1989) containing 75 mg/l ampicillin and 25 mg/l chloramphenicol. IPTG was added to a final concentration of 0.4 mM at ~0.5–0.6 OD_{600} (optical density at 600 nm) and the cultures were shaken for a further 2.5 h. Cells were harvested by centrifugation and resuspended in 20 ml of 50 mM Tris–HCl and 2 mM EDTA, pH 8.0. After two freeze–thaw cycles, $MgCl_2$ and DNase I were added to the viscous suspension to a final concentration of 20 mM and 6 mg/l, respectively. After centrifugation (5000 *g* for 10 min), the pelleted inclusion bodies were purified

further as described (Nagai and Thøgersen, 1987). The enriched inclusion bodies were dissolved in a denaturing solution (6 M guanidinium hydrochloride, 0.1 M Na-phosphate, 10 mM Tris–HCl, 10 mM 2-mercaptoethanol, pH 8.0). Metal-affinity chromatography using Ni-NTA resin (Qiagen) was attempted but neither subunit showed specific binding, so the solubilized subunit preparations were individually purified by gel filtration (Sephacryl S-300, 1.5×90 cm column) in denaturing solution. Fractions were analysed by SDS−10% PAGE and the purest fractions were pooled. Equimolar amounts of the two subunits were mixed in denaturing buffer and the solution was dialysed against refolding buffer (500 mM NaCl, 50 mM Tris−HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.2 mM EDTA, 2.5 mM PMSF, 1 μM leupeptin and 1 μM pepstatin) at 4°C. The dialysed sample was centrifuged at 14 000 r.p.m. (Eppendorf microcentrifuge) for 10 min and the supernatant was used, either directly or after dsDNA affinity chromatography and glycerol gradient centrifugation, for DNA binding and helicase assays.

Other procedures

Affinity labelling of HDH II with [α-^{32}P]ATP was performed using 200 ng of pure HDH II, as described previously (Tuteja *et al.*, 1993). DNA topoisomerases were assayed according to Kaiserman *et al.* (1988), except that the plasmid DNA used was Bluescript containing a cDNA insert of 1 kb human cGMP phosphodiesterase (Tuteja *et al.*, 1990a). DNA ligase activity was checked, as described previously (Tuteja *et al.*, 1990b). Nicking activity was assayed as described by Hughes *et al.* (1989). The DNA polymerase was assayed as described by Ottiger and Hübscher (1984). Protein concentration was determined using the protein assay kit of Bio-Rad. SDS−PAGE was performed by the method of Laemmli (1970), followed by silver staining with the Bio-Rad kit.

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