The Ku antigen consists of two subunits of 70 and 83 kDa and is endowed with both duplex DNA end-binding capacity and helicase activity (human DNA helicase II). HeLa Ku can be isolated from in vitro cultured human cells uniquely as a heterodimer, and the subunits can be separated by electrophoresis only under denaturing conditions.

To dissect the molecular functions of the two subunits of the heterodimer, we have cloned and expressed their cDNAs separately in Escherichia coli. The two activities of Ku (DNA binding and unwinding) were reconstituted by mixing and refolding both subunits in equimolar amounts (Tuteja, N., Tuteja, R., Ochem, A., Taneja, P., Huang, N-W., Simoncits, A., Susic, S., Rahman, K., Marusic, L., Chen, J., Zang, J., Wang, S., Pongor, S., and Falaschi, A. (1994) *EMBO J.* 13, 4991–5001).

Renaturation of the separate subunits can be achieved in the presence of a synthetic solubilizing and stabilizing agent, dimethyl ethylammonium propane sulfonate (NDSB 195). The helicase activity of the Ku protein resides uniquely in the 70-kDa subunit, whereas the DNA end-binding activity can be reconstituted only through renaturation of the two subunits in the heterodimeric form and is practically absent in the separate subunits. The 83-kDa subunit, when refolded in the absence of the 70-kDa subunit, forms homodimers unable to unwind DNA and bind duplex ends. The three separate species (heterodimer, 70-kDa subunit, and 83-kDa subunit homodimer) all have ssDNA-dependent ATPase activity.

The Ku autoantigen was originally isolated as a nuclear protein recognized by the sera of lupus erythematosus patients (1); it is a heterodimer made of two subunits that have been ascribed slightly different molecular masses in different labs; in this work we shall define them by the molecular masses calculated from the straight amino acid sequence, namely 83 and 70 kDa. Ku has the ability to bind specifically to the ends of duplex DNA and then slide into the duplex to form a structure similar to beads on a string (2). This molecule is essential for the recombination events necessary for the rearrangement of the immunoglobulin genes (V(D)J recombination) as well as for the repair of double strand DNA breaks caused by x-ray damage. Cell lines bearing mutations affecting the 83-kDa subunit appear to be deficient in both of these properties as well as in the duplex DNA end-binding ability of the extracted nuclear proteins (3). Accordingly, Ku83-deficient mice exhibit severe combined immunodeficiency due to T and B lymphocyte arrest at early progenitor stages (3–4). Furthermore, Ku has also been reported to be a substrate as well as a cofactor of the DNA-dependent protein kinase, which is also essential for the V(D)J recombination and x-ray repair processes (5–7).

In the past, we showed that Ku is also endowed with an ATP-dependent DNA helicase activity denominated human DNA helicase II (HDH II)\(^1\), probably located on a different moiety of the molecule than the one involved in duplex DNA binding (8). Furthermore, after cloning of the cDNAs of the separate subunits, we could obtain the proteins in pure form and renature the heterodimer, partially reconstituting both DNA binding and DNA unwinding activities. Under those conditions, it was not possible to separately renature the two subunits, since the 73-kDa one could not be solubilized in the absence of the other one.

In this work, we used a novel molecule designed to facilitate protein solubilization (9–10) and renaturation (11). This has allowed the separate refolding of either subunit; it was thus possible to study on which subunit(s) the two measurable functions of Ku reside.

### EXPERIMENTAL PROCEDURES

#### Buffers—All the buffers except buffer G contained 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μM pepstatin, and 1 mM sodium metabisulfite. Buffer G contained 6 mM guanidinium hydrochloride, 100 mM sodium dihydrogen phosphate, 10 mM Tris-HCl, pH 8, 10 mM β-mercaptoethanol. Buffer R contained 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM MgCl\(_2\), and 0.2 mM EDTA. Buffer C contained 20 mM Tris-HCl, pH 7.5, 5% glycerol, 3 mM MgCl\(_2\), 100 mM NaCl, and 0.2 mM EDTA.

#### Expression and Purification of Recombinant HDH II/Ku—The cloning of the cDNAs of the HDH II/Ku subunits was described previously (8). *Escherichia coli* cells (strain BL 21 (DE3)(pLysS)) transformed with the respective plasmids for the 70- or 83-kDa subunits of HDH II/Ku were grown in LB medium (12) containing 75 mg/l ampicillin and 25 mg/l chloramphenicol with vigorous shaking to an optical density of 0.5 at 600 nm. Protein expression was induced by the addition of isopropyl-β-thio-galactoside to a final concentration of 0.4 mM, and the cultures were shaken for a further 2.5 h. Cells were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, pH 8, 2 mM EDTA). After two freeze-thaw cycles, the extremely viscous solution was fluidified by sonication and centrifuged.

The pelleted inclusion bodies were solubilized in buffer G (denatur-
ing buffer) and purified by gel filtration on Sephacryl S300 (Pharmacia Biotech, Uppsala, Sweden) at 25 °C in the same buffer to eliminate possible low molecular mass contaminants of bacterial origin and renatured in the presence of dimethylammonium propane sulfonate (see “Results”). The refolded separate subunits were adjusted by the composition of buffer C and applied separately onto an MonoQ HR5/5 (1 ml) column equilibrated on fast protein liquid chromatography (Pharmacia) in buffer C.

Purification of the refolded structures was followed by monitoring the DNA unwinding activity for the 70-kDa subunit and by DNA binding and helicase assays for the recombinant heterodimer, whereas for the 83-kDa subunit, purification was followed by SDS-PAGE analysis. Purification and refolding of the separate subunits of the recombine heterodimer, whereas for the 83-kDa subunit, purification was followed by SDS-PAGE analysis.

Electrophoretic Mobility Shift, Helicase and ATPase Assays—DNA gel retardation experiments were performed on a 32P-5′ end-labeled duplex of 25 base pairs (5′-GATCTGACAACTGAGGATC-3′) and its complementary sequence when appropriate) essentially as described (13) with some modifications. The 20-μl reaction mixture contained 20 mM Tris, pH 8, 1 mM MgCl₂, 4 mM ATP, 60 mM KCl, 8 mM dithiothreitol, 4% (w/v) sucrose, 80 μg/ml bovine serum albumin, ~0.05 pmol of 32P-labeled DNA probe (generally 10,000–30,000 cpm) and the enzyme fractions. After incubation of the reaction mixture for 30 min at room temperature, the products were separated on a 5% nondenaturing PAGE in buffer containing 45 mM Tris, 45 mM boric acid, and 1 mM EDTA at 4 °C. After electrophoresis, the gel was dried, and bands were visualized by autoradiography and quantified after excision in a β counter.

The helicase assay consisted of the same components as the gel shift assay in a 10-μl reaction mixture except for the DNA substrate, which was a 32P-5′ end-labeled 47-mer oligonucleotide annealed to M13 ssDNA in its central 17 nucleotides, yielding a partial duplex with 5′ and 3′ tails of 15 nucleotides each. The sequence of this oligonucleotide as well as the structure of the substrate has already been reported (14). About 0.005 pmol of the substrate (generally ~5000 cpm) was used in each reaction mixture. After incubation with the enzyme fractions for 30 min at 37 °C, the reaction was terminated by the addition of 10 mM EDTA, 5% glycerol, 0.3% SDS, and 0.3% bromphenol blue (final concentrations). The products were analyzed by electrophoresis on a 12% nondenaturing PAGE in buffer containing 90 mM Tris, 90 mM boric acid, and 0.3% SDS, and 0.3% bromphenol blue (final concentrations). The products were analyzed by electrophoresis on a 12% nondenaturing PAGE in buffer containing 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, and helicase activity was visualized as described earlier (15). We define 1 unit of helicase activity as the amount of enzyme that retards 1 fmol of DNA probe in a gel retardation assay. ATPase assays were performed as already described (14), with minor modifications in the final concentrations of ATP (100 μM) and γ-32P (33 μCi) used.

**RESULTS**

Purification and Refolding of the Separate Subunits of Recombinant HDH II/Ku—The cloning and expression of the separate subunits of HDH II/Ku in E. coli were carried out as already described (8). The recombinant proteins accumulated in inclusion bodies were isolated by standard methods (Ref. 16; see also “Experimental Procedures”) and further subjected separately to gel filtration on Sephacryl S300 resin. Earlier attempts to renature the 70-kDa subunit separately by dialysis were unsuccessful, since it showed an intrinsic tendency to precipitate. However, when the separate subunits of HDH II/Ku (50 ml of Sephacryl S300-purified fractions in buffer G) as well as an equimolar mixture of these were treated individually by the addition of the nondenaturing solubilizing and stabilizing agent NDSB 195 (dimethylylammonium propane sulfonate) (9–11) at a final concentration of 0.2 mM and dialyzed at 4 °C versus eight changes of 2,000 ml of buffer R, each of the separate subunits remained in solution during dialysis, and renaturation could thus be achieved. (An earlier attempt at refolding using NDSB 195 at a final concentration of 0.1 M had resulted in slight precipitation of the separate subunits during dialysis). NDSB is a nondenaturant with low molecular mass (see “Results”). The refolded separate subunits were adjusted by the composition of buffer C and applied separately onto an MonoQ HR5/5 (1 ml) column equilibrated on fast protein liquid chromatography (Pharmacia) in buffer C.

Purification of the refolded structures was followed by monitoring the DNA unwinding activity for the 70-kDa subunit and by DNA binding and helicase assays for the recombinant heterodimer, whereas for the 83-kDa subunit, purification was followed by SDS-PAGE analysis.

**Fig. 1.** Purification of the separate recombinant HDH II/Ku subunits. Panel A, expression of Ku subunits in BL 21 (DE3) (pLysS) E. coli. Total cell extracts corresponding to 50-μl uninduced or isopropyl-1-thio-β-D-galactopyranoside (IPTG)-induced cultures were analyzed on SDS-10% polyacrylamide gel. Lane 1, pET6b Ku70 uninduced; lane 2, pET6b Ku70 induced; lane 3, pET5a Ku83 uninduced; lane 4, pET5a Ku83 induced; lane 5, molecular size markers (low range (M(l)r)) with corresponding mass indicated on the right-hand margin. Proteins are visualized by Coomassie Brilliant Blue staining. The positions of the recombinant Ku (rKu) moieties are indicated on the left side. Panels B and C, SDS-PAGE and Western blot analyses of refolded recombinant Ku and subunits. Approximately 5 μg of each protein sample were analyzed on SDS-10% polyacrylamide gel and visualized by Coomassie Brilliant Blue staining (panel B) or revealed by Western blotting (panel C). Lane 1, low range molecular mass markers; lanes 2 and 3, rKu70-Ku70 subunit before and after MonoQ chromatography, respectively; lanes 4 and 5, rKu70-Ku70 subunit before and after MonoQ; lane 6, recombinant heterodimer; lane 7, high range (hr) molecular mass markers. The protein bands with lower masses are all recognized by the respective antibodies. The molecular masses of the two groups of mass markers is indicated on the left.

for 20 min, and the supernatants were used either directly or after one further purification step for DNA binding and helicase assays.
The refolded separate subunits were individually subjected to a further purification step by MonoQ anion exchange chromatography. After extensive washing of the column, bound material was eluted in each case with 20 column volumes of 0.1–1 M NaCl linear gradient. In each case the subunit eluted at approximately 0.25 M salt.

The reconstituted heterodimer was further purified by double strand DNA-Sepharose affinity chromatography as already described (17–18). Fig. 1, panel A, shows an SDS-PAGE analysis of the noninduced and induced bacterial strains before gel filtration, panel B, shows the SDS-PAGE, and panel C, shows the Western blot analyses of the various refolded recombinant HDH II/Ku species before and after their final steps of purification.

The Helicase Activity of HDH II/Ku Resides in the 70-kDa Subunit—The renatured separate subunits of HDH II/Ku were assayed for the in vitro activities (band shift and helicase) to determine whether these could be associated with either subunit. As shown in Fig. 2, panel A, the 70-kDa subunit possesses a helicase activity comparable to that of the heterodimer, whereas the 83-kDa subunit shows no detectable helicase activity. Neither subunit shows any appreciable capacity to retard the mobility of duplex DNA, indicating that neither of them alone has the ability of the native Ku to bind to the duplex ends (Fig. 2, panel B). Conversely, the 70-kDa subunit alone maintains a measurable affinity for ssDNA comparable to that of native and recombinant Ku (Fig. 2, panel C), in agreement with the maintenance of the helicase activity in this subunit, since most known helicases bind initially to the single strand portion of the substrate. These findings suggest that, whereas the helicase activity ascribed to HDH II/Ku resides only in the 70-kDa subunit, the DNA end-binding capacity requires the presence of both subunits in the heterodimeric form. A comparative quantitation of the activities of the different forms of HDH II/Ku is reported in Table I. Before analyzing in detail the catalytic and binding constants for functional properties of the different molecular forms, we investigated their subunit composition.

Quaternary Structure of the Renatured Molecules—We determined the native molecular mass of the refolded separate subunits of HDH II/Ku as well as that of a post-refolding equimolar mixture of these by a combination of glycerol gradient sedimentation (15–35%) and gel filtration in buffer C as described by Siegel and Monty (19). We also determined whether the heterodimer could be reconstituted by mixing equimolar amounts of the separately refolded subunits. As shown in Figs. 3 and 4, the helicase activity of the renatured 70-kDa subunit alone showed a native molecular mass correspondent to the one observed in SDS-PAGE; in the post-refolding equimolar mixture, the same molecular mass was measured for helicase activity, whereas in the reconstituted heterodimer, the activity showed a molecular mass corresponding to that of the sum of the molecular mass values of the two subunits (Fig. 3). SDS-PAGE analysis of the gel filtration experiments showed that the 83-kDa subunit, whether renatured alone or in the post-refolding mixture, eluted at a volume close to that of the reconstituted heterodimer (Fig. 4). We therefore conclude that the refolded 70-kDa subunit of HDH II/Ku remains as a monomer in solution, even in the presence of equimolar amounts of the other subunit and that the heterodimer cannot be reconstituted by...
Properties of the HDH II/Ku Subunits

mixing and incubating equimolar amounts of the renatured subunits; furthermore, the 83-kDa subunit, when renatured alone, forms homodimers that do not spontaneously exchange with the renatured 70-kDa subunit added subsequently (see Fig. 4).

A more sensitive comparative quantitative analysis of the functional properties in vitro of the different molecular forms was then performed.

Substrate and Protein Concentration Dependence for the Various Forms and Activities of HDH II/Ku and Subunits—The various forms of HDH II (HeLa-purified heterodimer, reconstituted recombinant heterodimer, and recombinant 70-kDa subunit) were assayed for helicase activity in the presence of increasing amounts of substrate. Fig. 5, panel A shows a direct plot of DNA unwinding against substrate concentration. Fig. 5, panel B, shows the double-reciprocal plot to determine the $K_m$ values relative to these enzyme species. It appears that these three enzyme species have the same affinity for the helicase substrate ($K_m$ of 0.5 nm) but different $V_{\text{max}}$ values when expressed as mmol of substrate unwound × mol$^{-1}$ of enzyme × min$^{-1}$ (namely, 2.1 for the natural Ku form, 1.7 for the recombinant form, and 0.4 for the 70-kDa subunit).

The DNA binding ability, as assayed by electrophoretic mobility gel retardation, is essentially observed only for the native heterodimeric form of HDH II/Ku and, albeit at a markedly reduced level, for the recombinant heterodimer. The 70-kDa subunit exhibits only traces of DNA binding at very high protein/DNA ratios, whereas the 83-kDa subunit shows no detectable DNA binding in the range of assayed protein concentrations. Conversely, the ability to bind ssDNA, that in the native form is approximately one-hundredth that for duplex DNA, is reconstituted almost completely in the recombinant heterodimer and (not surprisingly, as seen above) in the 70-kDa subunit, i.e. the moiety where the unwinding capacity resides.

We then determined the protein concentration dependence for the DNA binding activity of the two heterodimeric forms (HeLa-purified and recombinant) of HDH II/Ku. Fig. 6 shows a linear dependence of DNA binding on protein concentration of up to 5 ng for the HeLa-purified enzyme, with an apparent $K_d$ for this species of approximately 1.0 nm. The recombinant heterodimer showed, as pointed out earlier, only 1.8% of the DNA binding activity observed for the HeLa-purified enzyme, and the estimated value of $K_d$ for the reconstituted molecule is

![Glycerol gradient sedimentation analysis of renatured forms.](image)

**TABLE I**

Quantitation of the in vitro activities of recombinant HDH II/Ku heterodimer and subunits and comparison with those of HeLa-purified heterodimer.

| Species assayed | DNA binding | Helicase activity | ssDNA-dependent ATPase |
|----------------|-------------|------------------|------------------------|
|                | 25-mer duplex | 25-mer single strand | | |
|                | units/mg$^a$ | % relative to HeLa Ku | units/mg$^a$ | % relative to HeLa Ku | units/mg$^a$ | % relative to HeLa Ku |
| HeLa HDH II/Ku | 3.6 × 10$^6$ | 100 | 1.9 × 10$^4$ | 100 | 1.7 × 10$^5$ | 100 |
| recombinant HDH II/Ku | 6.5 × 10$^4$ | 1.8 | 1.4 × 10$^4$ | 72 | 1.5 × 10$^5$ | 88.2 |
| recombinant Ku 83 | <10 | 3 × 10$^2$ | 1.4 | <10 | <0.05 | 3.6 |
| recombinant Ku 70 | ~780 | ~0.02 | 1.4 × 10$^4$ | 72 | 7.6 × 10$^4$ | 44.7 |

| Enzyme species | DNA binding | Helicase | ssDNA-dependent ATPase |
|----------------|-------------|----------|------------------------|
|                | $K_d$, 25-mer duplex | $K_d$, 25-mer single strand | $K_m$, helicase substrate | $V_{\text{max}}$, helicase substrate | $K_m$, ATP | $V_{\text{max}}$, ATP |
|                | $\mu M$ | $\mu M$ | $\mu M$ | mmol unwound/30 min (mol enzyme × min) | $\mu M$ | mol hydrolyzed/30 min (mol enzyme × min) |
| HeLa HDH II/Ku | 0.001 | 0.06 | 0.5 | 2.1 | 11.7 | 5.9 |
| recombinant HDH II/Ku | 0.05 | 0.07 | 0.5 | 1.7 | 7.7 | 4.8 |
| recombinant Ku 83 | $>20$ | 10 | 10 | $>1 \times 10^4$ | <1 × 10$^{-4}$ | ND$^a$ |
| recombinant Ku 70 | ~10 | 0.14 | 0.4 | 0.7 | 0.3 |

$^a$ Not determined.

![Species assayed](image)
Dependence of DNA Unwinding on ATP—The amount of DNA unwound in the presence of increasing concentrations of ATP in the standard assay condition is shown in Fig. 7 and reported in double-reciprocal form in Fig. 8. We can estimate $K_m$ values for ATP of 11.7 mM for the HeLa-purified enzyme, 7.7 mM for the recombinant heterodimer, and 4.4 mM for the 70-kDa subunit. The observed differences in the affinities of these enzyme species for ATP are in agreement with the above reported differences in the $V_{\text{max}}$ of their respective DNA helicase activities.

DNA-dependent ATPase Activity of the Different Molecular Forms—The various species of HDH II/Ku as well as the separate subunits were assayed for ssDNA-dependent ATPase activity, as described under “Experimental Procedures,” in the presence of 100 $\mu$M ATP. For all the species tested, ATP hydrolysis was observed in the assay conditions, namely at the level of 1.12 $\mu$mol of ATP/mg for HeLa-purified Ku, 0.9 $\mu$mol/mg for recombinant Ku heterodimer, 0.3 $\mu$mol/mg for recombinant Ku 70, and 0.11 $\mu$mol/mg for recombinant Ku 83 (see also Table I). As shown above, ATP-dependent DNA-unwinding activity had been observed for all these species except the 83-kDa subunit. The presence of this functionally unexplained ATPase activity in the large subunit agrees with the described presence of a putative ATP binding site in the same molecule (8). Table II summarizes the catalytic and binding constants as a duplex and ssDNA-binding protein, helicase, and ATPase of the different forms of Ku.

DISCUSSION

The results obtained by this work show the possibility of uncoupling the two in vitro activities of HDH II/Ku described earlier (8). The properties of the refolded HDH II/Ku and of the two separate subunits show that the DNA-unwinding activity ascribed to the heterodimer resides in fact exclusively in the 70-kDa subunit. The DNA end-binding capacity, however, remains a prerogative of the HDH II/Ku heterodimer, suggesting that the presence of this molecular form is required to perform the reactions necessary for repair of double-strand breaks and V(D)J recombination. Whether this scenario represents the situation in vivo is hard to say from this study but certainly raises the speculation of a possible differential in vivo expression of these subunits according to the circumstantial cellular requirements for Ku. It should be remembered here that the genes for the Ku subunits are localized on different chromosomes (20) and that a number of reports have attributed different properties to either subunit of Ku, suggesting that the enzyme may not always exist in its heterodimeric form (21–22). Since the heterodimer has always been isolated from proliferating nuclei, not much is known about the nature and structure of this protein in other phases of the cell cycle. Furthermore, the 70-kDa subunit alone has been immunologically localized to the nucleoli and periphery of interphase nuclei (23). The presence of helicase activity in the 70-kDa subunit alone therefore offers additional circumstantial evidence of the possible independent in vivo existence of this subunit in certain conditions and of possible additional roles for this molecule in DNA metabolism.

The observation that the unwinding activity resides in the small subunit whereas the heterodimeric structure is required for duplex DNA end-binding confirms our previous inference (based on the lack of reciprocal inhibition of either activity by the substrate of the other one) that these two properties are
located in two different moieties of the Ku heterodimer (8).

Evaluation of the DNA unwinding at different substrate concentrations gave a $K_m$ value of 0.5 nM for the three active species of HDH II/Ku. Thus, the three species have the same affinity for the helicase substrate notwithstanding their relative conformational and supramolecular differences. The differences in the observed $V_{max}$ values are parallel with the different affinities for ATP, which is not a surprising observation considering that helicase activity strictly depends on ATP hydrolysis.

The fact that the ATPase activity of the 83-kDa subunit is not associated to any appreciable helicase activity leaves unexplained its possible functional significance, although similar cases have been reported (24). It is conceivable that in the heterodimeric form, the intrinsic ATPase function of the 83-kDa subunit may be the basis for the higher $V_{max}$ of the heterodimer as helicase (more than 4-fold) with respect to the 70-kDa Ku subunit alone. The likely absence of a helicase active center in the 83-kDa subunit makes its ssDNA-dependent ATPase activity a futile one, at least at first sight.

Contrary to the situation with the unwinding activity, the DNA binding capacity was restored by the renaturation procedure rather poorly for the heterodimer as well. In fact, in different preparations, we observed a significant variability in the extent of reconstitution of DNA end-binding capacity of Ku, reaching in some cases 20% that of the activity of the native Ku heterodimer purified from HeLa cells. Furthermore, this capacity proved also very labile upon storage at 4°C, much more than for native Ku. The reasons for this discrepancy (with respect to the satisfactory reconstitution of unwinding activity) may lie on the fact that 1) the helicase active center (and the ssDNA binding moiety) resides on a distinct position of the molecule with respect to the duplex DNA end-binding domain and 2) post-translational modifications of Ku operating in vivo may enhance and stabilize the duplex end-binding capacity.

The separate subunits showed only minimal or no duplex DNA binding ability, even with respect to the reduced reconstituted property of the heterodimer; in fact, only the small subunit showed a binding capacity of the order of 1% that of the reconstituted recombinant dimer. From our data it appears that the formation of a dimeric structure per se is not enough to confer the property of binding DNA, since the large subunit can form homodimers without reacquiring the ability to bind DNA. It appears therefore that the duplex DNA binding ability is an intrinsic property of a specific molecular environment produced by the formation of the heterodimer.

This observation may appear in contrast with previous reports from different laboratories including ours (18) that the separate subunits show ability to bind DNA probes in a South-Western type of assay, the 70-kDa subunit having greater affinity than the large one (25). On the other hand, the assay for electrophoretic gel retardation is a much more stringent one than the South-Western type of assay, the 70-kDa subunit having greater affinity than the South-Western, and it is likely that this discrepancy is purely apparent. In fact, if the interaction of the small subunit with DNA is measured also (see e.g. Wang et al. (26)) with other methods that, like the South-Western assay, do not allow a precise determination of the $K_d$ value, this molecule shows indeed a certain capacity of binding duplex DNA. The same
authors, on the other hand, confirm that the 70-kDa subunit does not cause any appreciable band shift on duplex DNA. Wu and Lieber (27), by a completely different approach (two-hybrid and biochemical analyses of the interactions of Ku 70 and 83 and of truncated forms of these molecules), arrive in fact to the same conclusions as this report. In their work, neither the 70- nor the 83-kDa subunit, when translated alone, is able to bind DNA in band-shift assay, whereas they do so when translated jointly. These authors are also able to ascribe to C-terminal regions of the two subunits the properties of heterodimer formation and DNA binding. It seems reasonable to conclude at this stage that neither subunit alone can efficiently bind duplex DNA.

These considerations do not apply to the ability to bind ssDNA, which is in all probability related (functionally and physically) to the unwinding capacity of Ku. In fact, the affinities of the recombinant heterodimer and of the 70-kDa subunit for ssDNA are quantitatively comparable to the reconstituted DNA-unwinding capacity of the same molecular forms (see Tables I and II). Also, the 83-kDa subunit homodimer main-

FIG. 7. Dependence of helicase activity on ATP concentration. Helicase assays were carried out in the presence of increasing amounts of ATP and 11 ng of enzyme. Panel A, recombinant 70-kDa subunit. Lane 1, no enzyme; lanes 2–9, assays with 0, 0.5, 1, 2, 3, 4, 5, and 6 mM ATP, respectively; lane 10, heat-denatured substrate. Panel B, HeLa Ku. Lane 1, no enzyme; lanes 2–8, assays with 0, 0.5, 1, 2, 3, 4, and 5 mM ATP, respectively; lane 9, heat-denatured substrate; lane 10, assay with 6 mM ATP. Panel C, recombinant Ku (rKu) heterodimer. Lane 1, no enzyme; lanes 2–7, assays with 0, 0.5, 1, 2, 3, and 4 mM ATP, respectively; lane 8, heat-denatured substrate; lanes 9 and 10, assays with 5 and 6 mM ATP, respectively.

tains a trace (small but significant) of ssDNA affinity that probably sustains the trace of ssDNA-dependent ATPase activity observed for this molecular form.

Structural data on Ku are not yet available, but the properties measured by biochemical means would favor the presence of some form of ring structure in the heterodimer able to bind to ends and then slide along the duplex DNA in a nearly irreversible way. This observation is in agreement with the wealth of data indicating a strong but promiscuous requirement of Ku for binding DNA ends, since the heterodimer can bind them irrespective of their DNA sequence or their chemical-detailed structure (5’ protruding, 3’ protruding, phospho-

FIG. 8. Determination of the $K_m$ for ATP for the helicase activity of the various forms of HDH II/Ku. Panel A, direct plot of dependence of helicase activity on ATP concentration determined from the data of the results shown in Fig. 6. The $K_m$ values that these enzymes species exhibit for ATP are calculated from the double-reciprocal plot in panel B.
Ku to bind frayed ends would fit very well with the function of Ku in the illegitimate recombination processes required for immunoglobulin gene maturation and for x-ray damage repair. Obviously, the presence of a helicase activity (active only on DNA partial duplexes and not on RNA-containing structures (8)) could fit very well also with these events, even though no evidence has been available so far in this sense. The production of mutations and deletions in either subunit and the study of the functional effects of these mutations on those cellular processes will shed more light in the future on the functional effects of these mutations on those cellular processes. Also, the relatively low capacity of DNA end-binding of the recombinant Ku (as well, of the effect of in vitro dependent protein kinase. phosphorylation of this molecule operated different ways in different moments of the cell cycle) by the course, as the helicase activity itself) may be modified (in different ways in different moments of the cell cycle) by the phosphorylation of this molecule operated in vivo by the DNA-dependent protein kinase.

Studies of the properties of partially deleted 70-kDa subunits and of the effect of in vitro phosphorylation of Ku on its functional activities are in progress.

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