Production and characterization of recombinant human Ku antigen

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ABSTRACT

Ku is an ubiquitous nuclear heterodimeric protein consisting of p70 and p86 subunits that binds double-stranded DNA termini and associates with chromosomes *in vivo*. It was originally described as an autoantigen in patients with certain autoimmune diseases. The individual subunits of Ku have been difficult to isolate from human cells without denaturation and attempts to produce functional recombinant Ku have been largely unsuccessful. Here, we utilize two recombinant baculoviral vectors that carry p70 or p86 cDNA and express the Ku subunits individually as well as assemble them into the complete Ku heterodimer. In an electrophoretic mobility shift assay, recombinant Ku binds to linear double-stranded DNA but not to supercoiled, nicked circular, nor linear single-stranded DNA. Neither subunit binds DNA by itself indicating that heterodimerization is essential for function. We also describe a simple purification method for the isolation of highly purified recombinant Ku using a hexahistidine tag. The baculovirus expression system provides a stable and efficient source of not only the p70 and p86 subunits but also the functional Ku heterodimer.

INTRODUCTION

Autoantibodies are found in the sera of patients with many systemic autoimmune diseases, several of which have served to distinguish various subsets of patients with distinct clinical syndromes. Several of these autoantibodies are directed against cellular components and many of molecules so identified bind DNA or RNA (1). While Ku autoantibodies were originally described in a patient with overlap syndrome (2), currently these autoantibodies are more commonly found in patients with systemic lupus erythematosus and mixed connective tissue disease (3–5).

There is abundant evidence that Ku is a DNA binding protein. It has been isolated as a nuclear factor (NFIV) from HeLa cells (6), and localized to the peripheral area of the nucleus and nucleolus often associated with active chromatin (7,8). In addition, the deduced amino acid sequence of the p70 subunit

contains periodic leucine repeats with considerable homology to some well characterized transcription factors (9,10).

Ku shares amino acid sequence homology with (or may be identical to) several proteins. For example, PSE1 which binds to the PSE element of the U1snRNA promotor and upregulates U1RNA transcription *in vitro* is immunologically cross reactive with Ku and limited amino acid sequence comparison shows them to be identical (11,12). TREF, which binds the transferrin promotor, is also immunologically similar to Ku and shares some amino acid sequence identity with PSE1 (11). A second form of Ku, 'Ku-2', was isolated from Daudi cells and has been shown to bind the octamer motif (13). The amino acid sequence of 'Ku-2' is slightly different from Ku, but its DNA sequence has not been reported. Therefore, the relationship between 'Ku-2' and Ku is still unclear. All of the above proteins (PSE1, TREF, 'Ku-2') may be identical to Ku.

Although the DNA binding properties of Ku have been extensively studied, controversy remains as to its binding specificity. Thus, while Ku has considerable affinity for linear double-stranded DNA (dsDNA), its affinity for many DNA and RNA structures is not entirely clear and there are several contradictory reports (13–21). The DNA binding domain has been located to the p70 subunit and it has been suggested that it contains a helix-turn-helix DNA binding motif (14,18,19). These conclusions were primarily based on a southwestern analysis where p70 and p86 were separated by SDS–PAGE and renatured on nitrocellulose membranes. However, Griffith *et al.*, (17) using an electrophoretic mobility shift assay (EMSA), reported that both subunits were required for DNA binding, suggesting that the heterodimeric form may be required for DNA binding under more physiologic conditions. On the other hand, Messler *et al.*, (22) showed that the C-terminal portion of the p70 fragment bound to specific DNA by itself.

The DNA sequence specificity of Ku has been examined in considerable detail. We originally isolated Ku because it bound to an octamer (ATGCAAAT) affinity column (23). Other investigators have shown that Ku binds to varying sequence-specific DNA affinity columns (11,20,22–28). Additionally, Ku related factors are reported to give clear footprints with the octamer motif (13) and the proximal element of the U₁RNP promotor (11). Ku has been reported to have affinity for AT-

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rich dsDNA termini (15), however, there is considerable evidence suggesting that Ku shows no sequence specificity when analyzed by EMSA (16,29). These apparent contradictions may be the result of different sources of Ku and different purification methods. They could be the result of contaminating nuclear proteins associated with Ku during purification as there is abundant evidence that Ku associates with several different nuclear proteins, some of which are known to bind specific DNA elements (15,26–28). Additionally, the two subunits have been difficult to purify separately in native form because of their high affinity for each other (23). Therefore, a stable system to obtain the Ku subunits seems essential to further detail the interesting biology of this molecule (28).

There have been several attempts to express recombinant Ku but it appears unstable in bacterial expression systems. Reeves and Stoecker (9) reported that the full-length p70 subunit was expressed in only trace amounts in bacteria. Yaneva *et al.*, described a similar phenomena with the p86 subunit (30). Here, we engineered Ku into a baculovirus expression system and successfully produced significant quantities of recombinant heterodimeric protein. In addition to documenting that the recombinant product is full-length, we show that it binds DNA under physiological conditions. Finally, we confirm that heterodimerization is necessary for Ku to bind linear dsDNA.

MATERIALS AND METHODS

Cells, DNA, antisera and enzymes

The *Spodoptera frugiperda* cell line Sf9 (ATCC CRL1711) was grown in TNM-FH medium (Sigma, MO)/10% FCS and antibiotics/antimycotics at 27°C. Linear AcMNPV DNA and the transfer vector pBlueBac2 were obtained from Invitrogen (San Diego, CA). A pBluescribe vector that carried the full-length p86 subunit cDNA was obtained from Dr Mariana Yaneva, Baylor College of Medicine, Houston, TX (30), and a p70 subunit cDNA and recombinant virus (AcNPV2.1) was obtained from Dr Bellur S. Prabhakar, University of Texas Medical Branch, Galveston, TX (18). Human anti-Ku antisera that only reacted with the p86 subunit (SG) or that reacted with both subunits (IH) were obtained from Dr Morris Reichlin, Oklahoma Medical Research Foundation, Oklahoma City, OK. A 48 base oligonucleotide, BX-A (5'-TAAGATGTACCCTGTCTTCTT-AATTGTACCCTCAGGTGAGTCTATGGT-3') and BX-B (antisense of BX-A) was synthesized and annealed into dsDNA (BX), then purified from an acrylamide gel. To make nicked circular DNA, 10 ng/μl pBluescript was digested with 5×10^{-5} u/μl DNase I in 10 mM Hepes pH 7.9, 10 mM MgCl₂ for 15 min at 30°C and the digestion stopped with 50 mM EDTA on ice. After digestion, form II DNA was purified from an 0.8% agarose gel (31,32).

Construction of recombinant baculoviruses

A 3.4 kb *EcoRI* p86 cDNA fragment and the transfer vector pBlueBac2 digested with *Bam*HI were blunt-ended with the Klenow fragment and ligated with T₄ ligase. The recombinant vector (pBB2-86Ku) was picked by colony hybridization with a ³²P-labeled p86 cDNA probe and the orientation of the insert was confirmed by restriction digest analysis. For purification, the hexa-histidine tag was added to the c-terminus of p70 cDNA by PCR, and cloned into *Bam*HI site of pBluescript KS (Stratagene, CA) (pBC-70tH⁶) (33). The PCR product was confirmed by DNA sequencing. Then the 1.9 kb tagged p70

cDNA fragment from pBC-70tH⁶ was subcloned into the pBlueBac2 transfer vector (pBB2-70tH⁶).

Transfection and isolation of recombinant virus

Linear wild type AcMNPV (0.5 μg) and 2 μg of pBB2-86Ku were cotransfected into Sf9 cells (2×10^6 cells in a 25 cm² flask) using calcium phosphate. p86cDNA was inserted into the AcMNPV genome by homologous recombination (34). After 5 days, the supernatant containing recombinant virus (VBB2-86Ku) was harvested. During plaque purification, X-gal (150 μg/ml) was added to assist in the identification of recombinant plaques which became blue after 5–7 days. Expression of recombinant protein was verified by Western blotting with whole cell extracts from infected cells and human anti-Ku antiserum. The recombinant virus which carried the hexa-histidine tagged p70 cDNA was produced by the same procedure (VBB2-70tH⁶).

Production and preparation of recombinant proteins

Sf9 cells (4×10^5 cells/ml) were infected with one or both viruses at 20-fold multiplicity of infection (MOI) and then cultured in TNM-FH/10%FCS for 63–72 h. In the case of [³⁵S]methionine labeling of recombinant protein, cells at 48 h post-infection were washed with phosphate buffered saline (PBS) and cultured in methionine deficient Grace's medium (Sigma) for 30 min. Cells were then cultured in 100 μCi/ml TRAN35S-LABEL (ICN Biochemical, Inc.) containing medium for 15 h.

To prepare proteins, 1×10^7 cells were harvested, washed and lysed in 1 ml 40 mM Hepes pH 7.9, 1 mM EDTA, 2 mM dithiothreitol (DTT), 0.1% Nonidet P-40, 1 μg/ml Leupeptin, 1 μg/ml Pepstatin A, 1 mM PMSF by freeze & thaw and sonication. After removal of non-soluble cell debris by centrifugation, proteins were precipitated by 30–70% ammonium sulfate and resuspended in 5 ml of 20 mM Hepes pH 7.9, 0.1 M KCl, 0.2 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, 20% glycerol, 1 μg/ml Leupeptin, 1 μg/ml pepstatin A, 1 mM PMSF, and dialyzed.

Purification of heterodimeric Ku

Sf9 cells co-infected with both VBB2-86Ku and VBB2-70tH⁶ were harvested as above. After ammonium sulfate precipitation, proteins were resuspended and dialyzed into 50 mM Na-phosphate pH 8.0, 500 mM NaCl, 10% glycerol. For purification, an immobilized metal ion affinity chromatography (IMAC) and DNA affinity column were utilized. First, Ni-NTA-agarose (Qiagen) was equilibrated with sample buffer and the protein sample was applied to the column. The column was then washed with 50 mM Na-phosphate pH 7.0, 500 mM NaCl, 10% glycerol. The tagged proteins were eluted with elution buffer (50 mM Na-phosphate pH 5.0, 500 mM NaCl, 10% glycerol). Fractions were collected in a tube containing 1/10 volume of 1 M Hepes pH 7.9. Second, for the random DNA column, salmon sperm DNA was fragmented by sonication as well as digestion with *Hha*I and *Sau*3AI. These random DNA fragments were coupled using cyanogen bromide activated sepharose 4B (Sigma). The protein sample from the Ni column was dialyzed against buffer D (20 mM Hepes pH 7.9, 150 mM KCl, 0.5 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, 20% glycerol, 1 mM PMSF), and applied to the random DNA column which was equilibrated with buffer D. The column was washed with buffer D (300 mM KCl) and the Ku heterodimer was eluted by buffer D made in 500 mM KCl.

Western blot analysis

SDS-PAGE was performed as described (35). Cells were washed, suspended in PBS, and lysed in Laemmli sample buffer. Proteins were separated on an 8% polyacrylamide gel and stained with Coomassie blue. For Western blotting, after SDS-PAGE, proteins were transferred to PVDF membranes and detected by the anti-Ku antiserum. In the final step, ^{125}I -labeled secondary antibodies were used, and reactions were visualized by autoradiography.

Co-immunoprecipitation

Protocols were performed on ice or 4°C. Protein samples were prepared as follows: [^{35}S]-labeled 2×10^6 cells were harvested and washed three times with PBS, and suspended in 500 μl lysis buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 $\mu\text{g/ml}$ Leupeptin, 1 $\mu\text{g/ml}$ Pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF)). Suspended cells were frozen at -80°C and sonicated. Lysates were checked by microscopy. Non-soluble debris was removed by centrifugation.

For immunoprecipitation, 3 μl of each lysate was diluted to 100 μl with lysis buffer and precleared with 40 μl (50% slurry) protein A-agarose beads (Sigma, St Louis, MO). Protein A-agarose beads were then coated with anti-Ku antibodies in PBS/5% BSA and preincubated with unlabeled Sf9 cell lysate and then washed with PBS. The beads were added to the precleared lysates and incubated for 1 h. Immunoprecipitates were separated on SDS-PAGE, and then visualized by autoradiography.

Electrophoretic mobility shift assay

As a linear dsDNA probe, a 147 bp *NheI*-*StyI* fragment was prepared from p70 cDNA with the polymerase chain reaction

and restriction enzyme digestion. The probe was labeled with [^{32}P] dCTP (36).

A typical assay was performed as follows: Protein was incubated with labeled DNA for 30 min at room temperature in 20 μl of buffer D (20 mM Hepes pH 7.9, 0.15 M KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 1 mM PMSF). After incubation, DNA-protein complexes were analyzed on an acrylamide gel (4% polyacrylamide, 50 mM Tris-borate, 1 mM EDTA, 2% glycerol). In some cases competitor DNA was added to the reaction for 0–5 min prior to the addition of the labeled probe (37).

RESULTS

Expression of recombinant Ku subunits in Sf9 cells

Recombinant baculoviruses containing full-length p70cDNA or p86cDNA were prepared and expressed in Sf9 cells. Protein production was confirmed by SDS-PAGE and visualized by Coomassie staining (Figure 1A), and immunoblotting with anti-human Ku antibodies (Figure 1B). Maximum expression of recombinant protein occurred 60–72 h post-infection, with Ku subunits accounting for up to 60% of most lysates. The recombinant 70kD protein reacted with Ku antiserum in immunoblot. VBB2-86Ku infected Sf9 cells produced an 86 kD band that reacted with anti-Ku antiserum and lesser amounts of 66 kD and 62 kD materials that did not react (Figure 1). These were probably proteolytic products of p86 because they were not detected in wild type AcMNPV or p70 subunit expressing Sf9 cells and became prominent when infected cells were placed in long-term culture, or during several freeze & thaw cycles. However, the p70 and p86 subunits were stable when cell lysates were incubated at 37°C for 60 min with proteinase inhibitors (data not shown).

Figure 1. Expression of Ku p70 and p86 subunits in Sf9 cells. Cells were infected with AcNPV2.1, VBB2-86Ku and wild type AcMNPV, and harvested 68 h postinfection. After three washes with PBS, whole cell lysates were analyzed by SDS-PAGE. A) Coomassie blue staining. Lane 1, VBB2-86Ku infected Sf9 cells; lane 2, AcNPV2.1 infected Sf9 cells; lane 3, uninfected Sf9 cells. Location of molecular mass markers indicated on the left of each figure and recombinant Ku subunits (p70 and p86) are indicated on the left. * indicates the positions of the 62 and 66kD products of VBB2-86Ku infected Sf9 cells. B) Immunoblot with anti-human Ku (IH). Lane 1, HEP-2 cells; lane 2, VBB2-86Ku infected Sf9 cells; lane 3 AcNPV2.1 infected Sf9 cells.

Figure 2. Co-immunoprecipitation of p70 with p86 with anti-p86 antiserum. Cells were infected with one or both viruses and labeled with [^{35}S]methionine. Proteins were precipitated using anti-p86 antiserum (SG) coated protein A beads. Samples (lane 1–3, cell lysates; lane 4–8, immunoprecipitates) were analyzed on 8% SDS-PAGE. Lane 1, 5, AcNPV2.1 infected Sf9 cells; lane 2, 6, VBB2-86Ku infected Sf9 cells; lane 3, 8, co-infected Sf9 cells (viral mixture as in Figure 2A, lane 6); lane 4, non-infected Sf9 cells; lane 7, AcNPV2.1 and VBB2-86Ku infected cells were mixed, lysed and incubated for 1 h at room temperature. Positions of p70 and p86 are indicated.

Expression of heterodimeric Ku in Sf9 cells

Two protocols were tested to produce the Ku heterodimer. In the first protocol, the p70 and p86 subunits were expressed individually. After harvesting, the cells which expressed p70 and those which expressed p86 were washed three times with PBS, mixed in a single tube, and lysed. Thus, in this protocol, the subunits were mixed *in vitro*. In the second protocol, the viral stocks were mixed prior to infection. The Sf9 cells were infected with mixed viral stock and cultured as above. Thus, in this protocol, Sf9 cells were co-infected by the recombinant viruses and both subunits were expressed.

To analyze the assembly of the heterodimer, translated proteins were labeled with [³⁵S]-methionine during culture, and cell lysates were co-immunoprecipitated with anti-p86 antibody (Figure 2). In mixed cell lysates from the first protocol, only small amounts of the p70 subunit were co-immunoprecipitated

with the p86 subunit by anti-p86 antibody. When the infected cells were lysed separately and then mixed, the same result was obtained. However, in co-infected cells, p70 was co-immunoprecipitated with p86. A similar result was observed in co-immunoprecipitation with anti-p70 antibody (data not shown). Thus, p70 and p86 were efficiently assembled into the heterodimer only under co-infection/co-translation conditions. Because the deduced amino acid sequences of the two subunits contain almost the same number of methionine residues, we estimate that almost all of the Ku subunits were assembled into the heterodimer (lane 8).

Purification of recombinant Ku heterodimer

Hexahistidine, a metal affinity peptide, was placed on the c-terminus of p70 (VBB2-70tH⁶). For purification, tagged p70 and non-tagged p86 were co-infected and expressed in Sf9 cells using the same procedure as in Figure 2. In this system, heterodimeric Ku should be tagged with hexa-histidine provided from the p70 subunit. The tagged-recombinant Ku heterodimer was purified simply and efficiently by passing it over two affinity columns. Fractions from the purification steps are indicated in Figure 3. Proteins were first separated by metal affinity, and then, were purified by DNA affinity. To estimate protein recovery through this purification scheme we checked each step with Coomassie blue staining of SDS-PAGE, the final recovery was approximately 10%.

DNA binding activity of recombinant Ku subunits

The DNA binding properties of the individual Ku subunits were studied by an electrophoretic mobility shift assay (EMSA) (Figure 4). The band shift due to Ku was confirmed by 'super shift' assay with an anti-Ku antibody (data not shown). In Figure 4A, lane 2, heterodimerized recombinant Ku showed multiple prominent DNA-protein complex bands. As shown in Figure 4B, when large amounts of protein were added, the bands were shifted to higher positions in logarithmic proportion, indicating that Ku formed multimeric complexes with DNA. Neither p86 alone (Figure 4A, lane 3) nor p70 alone (Figure 4A, lane 4), nor the

Figure 3. Purification of recombinant Ku heterodimer. The production of recombinant virus VBB2-70tH⁶ which carries a hexahistidine tagged p70 subunit and purification sequences are described in Materials and Methods. Protein fractions from each step were analyzed on 8% SDS-PAGE and visualized by silver-stain. lane 1, 100 ng BSA; lane 2, co-infected Sf9 cell lysate; lane 3, proteins precipitated by 30–70% ammonium sulfate; lane 4, protein fraction from Ni-NTA-agarose; lane 5, protein fraction from random DNA column.

Figure 4. DNA binding activity of recombinant Ku subunits. Protein samples were prepared as described in Materials and Methods, and 20 ng of each sample was incubated with ³²P-labeled linear dsDNA for 30 min at room temperature, and analyzed on a 4% acrylamide gel. **A)** Dimerized subunits are required for DNA binding. Lane 1, free probe; lane 2, AcNPV2.1 and VBB2-86Ku co-infected cells; lane 3, AcNPV2.1 infected cells and VBB2-86Ku infected cells were mixed as Figure 2 lane 7; lane 4, AcNPV2.1 infected Sf9 cells; lane 5, VBB2-86Ku infected Sf9 cells; lane 6, wild type AcMNPV infected Sf9 cells; lane 7, Sf9 cells. **B)** Oligomeric binding of Ku to linear dsDNA. The protein sample from co-infected cells were used. The amount of protein indicated above each lane was added in the reaction mixture and analyzed. **C)** The recombinant Ku heterodimer which was purified in Figure 3 was tested as B. The arrowheads indicate positions of the Ku-DNA probe complex.

two proteins mixed after harvest (Figure 4A, lane 5) bind DNA. These results demonstrate that the p70 and p86 subunits do not by themselves bind DNA, and suggest that the DNA binding activity of Ku requires heterodimerization. Similarly, the tagged Ku heterodimer which was highly purified in Figure 3 showed an identical binding pattern as Figure 4B (Figure 4C).

Binding of recombinant Ku to DNA ends

To further investigate how Ku binds to DNA, we first tested its DNA end recognition properties. An EMSA of recombinant Ku using the same DNA probe as used in Figure 4 was performed and the circular pGEM-3Zf(+) plasmid or *Eco*RI digested plasmid was added as competitor. The competition efficiency of circular and linear dsDNAs are shown in Figure 5A. The result shows that circular dsDNA was 200–500 times less competitive than linear dsDNA.

To confirm that DNA binding was initiated at DNA ends, we used three different lengths of linear dsDNA competitor. In this experiment, the total amount of competitor DNAs are equal, and the molarity of DNA ends were varied. Figure 5B shows that high DNA end concentrations are more competitive. This result demonstrates that DNA binding by recombinant Ku is initiated at DNA ends.

Binding activity of recombinant Ku to single-stranded and nicked circular DNA

Finally, we examined whether Ku binds to single-stranded DNA (ssDNA) and nicked circular dsDNA. In Figure 5C, 48 bp sense (BX-A) and antisense (BX-B) oligonucleotides were annealed to dsDNA and we compared the competitive efficiency of various single-stranded oligonucleotides. We found that ssDNA was 200–500 times less competitive than dsDNA. On the other hand,

Figure 5. DNA bindings of recombinant Ku. **A)** 7 ng of recombinant Ku was incubated with various competitors; lanes 2–6, *Eco*RI digested plasmid pGEM-3Zf(+); lanes 7–11, circular pGEM-3Zf(+) as indicated above each lane for 5 min at room temperature, and then the same labeled probe as in Figure 4 was added for 30 min and analyzed on a 4% acrylamide gel. Lane P, free probe; lane 1, without competitor. The arrows indicate the positions of the Ku–DNA complex. **B)** 2 ng labeled dsDNA and 5 ng competitor were added simultaneously in reaction mixtures which contained 17.5 ng recombinant Ku and incubated at room temperature for 30 min. Linear dsDNA competitors were prepared from plasmid pBluescript that carried p70 cDNA with several restriction enzyme sites. Competitor DNAs are indicated above each lane S, 150 bp; M, 380 bp; L, 1300 bp. Lane P, free probe; lane 1, without competitor. The arrows indicate the positions of the Ku–DNA complex. **C)** Linear dsDNA (BX) and linear ssDNA (BX-A) were prepared as described in Materials and Methods. 17.5 ng recombinant Ku was incubated with a labeled linear dsDNA probe for 30 min at room temperature, varying concentrations of (BX) or (BX-A) as competitor were added 5 min prior to probe addition. Lane P, free probe; lane 1, without competitor. Lane 2–6, BX; lane 7–11, BX-A added as competitor. The amount of competitor is indicated above each lane. The arrows indicate the position of the Ku–DNA complex. **D)** The plasmid pBluescript was nicked and converted to relaxed circular DNA using DNaseI digestion. 7 ng recombinant Ku and 2 ng 147 bp linear dsDNA probe were incubated for 30 min at room temperature, varying concentration of supercoiled or nicked pBluescript were added in the reaction mixture 5 min prior to probe addition. Lane 2–6, supercoiled; lane 7–11, nicked pBluescript were added as competitor. The amount of competitor is indicated above each lane. Lane P, free probe; lane 1, without competitor.

when the supercoiled circular pBluescript was nicked, converted to form II by DNase I digestion, purified from an agarose gel and both forms added as competitor in an EMSA, the competitive efficiency of form II DNA was similar to form I DNA (Figure 5D). We also tested UV irradiated DNA and which was digested with T4 endonuclease V (38) (data not shown). None of these are competitive, indicating that Ku may not bind pyrimidine dimer nor their nicks. Taken together, these experiments demonstrate that Ku does not bind either nicked circular dsDNA nor linear ssDNA efficiently.

DISCUSSION

We have expressed the p86 and p70 subunits of human Ku in a baculovirus system, assembled them into heterodimeric form, and documented that the recombinant material binds linear dsDNA under physiologic conditions. The expressed p70 and p86 subunits react with anti-human Ku antibody. In SDS-PAGE, VBB2-86Ku infected Sf9 cells contained two additional bands (p62 and p66) which are likely proteolytic products of p86. There has been some suggestion that the Ku complex may have a third subunit (termed p60-65) which gradually appeared when purified samples were incubated for several hours (11). Quinn *et al.* have shown a modification of p86 antigenicity and the appearance of p60 when cells were exposed to various stimuli such as viral infection, PMA and CaPO₄ (39). While the relationship of our observation to the putative 'third subunit' of native Ku remains uncertain, our data are most consistent with the interpretation that the third subunit is a degradation product of p86.

In human cells, it is well known that the p70 and p86 are assembled into the heterodimer in the absence of DNA (3,8,40). Therefore, we first attempted to assemble Ku from the individual subunits. However, only Sf9 cells that were co-infected with viruses encoding both subunits assembled the Ku heterodimer. When cells were infected separately with p70 and p86 viruses and the cells mixed and lysed, little if any heterodimer was formed. Thus, we conclude that co-infection with both viruses in Sf9 cells mimics co-translation, and that co-translation is required for heterodimerization. Further, there have been several attempts to document that the p70 subunit has DNA binding activity (14,18,19,29) including reports that the renatured p70 subunit by itself binds DNA attached to nitrocellulose membranes (14,18,19). However, It has been shown by crosslinking that p86 subunit also attached to DNA (19), moreover, Griffith *et al.*, had observed that their *in vitro* translated Ku p70 and p86 subunits by themselves did not bind DNA in EMSA (17). Our observation showed that only the assembled Ku complex had DNA binding activity in native condition suggesting that heterodimerization is required for DNA binding.

Several reports document that Ku recognizes dsDNA ends *in vitro* (14-17). We assayed the DNA binding specificity of recombinant Ku and found it has a high affinity for linear dsDNA but not for circular dsDNA. Further, increases in DNA end concentration of competitor caused higher competitive efficiency in an EMSA (Figure 5). These results indicate that Ku recognizes dsDNA ends. In addition, Ku forms a multimeric complex with DNA (Figure 4B, C). These findings are in agreement with de Vries *et al.* (15) who proposed that Ku binds at dsDNA ends and then moves to an internal position, allowing several molecules of Ku to bind to a single DNA molecule. However, it has been observed by laser scanning microscopy that Ku is associated with

chromosomes *in vivo* (7), and is released from nuclei by DNAase I digestion (8). This indicates that Ku associates with chromosomal DNA *in vivo*. While dsDNA ends are rare and transient *in vivo*, we have no evidence that Ku has endonuclease activity. While there are large amounts of Ku in the nucleic of all cells, it must have little opportunity to bind DNA in the nucleus (14). Therefore, the possibility still exists that other DNA conformations play a role in the binding of Ku *in vivo*. If so, Ku's strong DNA end binding property may obscure its authentic DNA binding specificity. In our hands, Ku has 200-500 times less affinity for linear ssDNA and nicked circular dsDNA than linear dsDNA (Figure 5C, D). These affinities were almost the same as supercoiled DNA, and are probably not candidates for the Ku binding initiation structure.

Recently, there have been two reports that the p350 catalytic component of a DNA-dependent protein kinase (DNA-PK, template associated protein kinase) physically associates with Ku, binds DNA, and that the kinase activity requires Ku (27,28). Additionally, it has been shown that DNA-PK is activated by linear dsDNA but not by ssDNA, supercoiled nor nicked circular DNA (28). These findings are compatible with the DNA binding properties reported here for recombinant Ku. Furthermore, it is known that Ku associates with several other nuclear proteins (15,26-28) and may also participate in their function. These results suggest that Ku may be a multifunctional or multiregulatory nuclear protein.

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