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Protein-Protein and Protein-DNA Interaction Regions within the DNA End-Binding Protein Ku70-Ku86

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DNA ends are generated during double-strand-break repair and recombination. A p70-p86 heterodimer, Ku, accounts for the DNA end binding activity in eukaryotic cell extracts. When one or both subunits of Ku are missing, mammalian cells are deficient in double-strand-break repair and in specialized recombination, such as V(D)J recombination. Little is known of which regions of Ku70 and Ku86 bind to each other to form the heterodimeric complex or of which regions are important for DNA end binding. We have done genetic and biochemical studies to examine the domains within the two subunits important for protein assembly and for DNA end binding. We found that the C-terminal 20-kDa region of Ku70 and the C-terminal 32-kDa region of Ku86 are important for subunit-subunit interaction. For DNA binding, full-length individual subunits are inactive, indicating that heterodimer assembly precedes DNA binding. DNA end binding activity by the heterodimer requires the C-terminal 40-kDa region of Ku70 and the C-terminal 45-kDa region of Ku86. Leucine zipper-like motifs in both subunits that have been suggested as the Ku70-Ku86 interaction domains do not appear to be the sites of such interaction because these are dispensable for both assembly and DNA end binding. On the basis of these studies, we have organized Ku70 into nine sequence regions conserved between *Saccharomyces cerevisiae*, *Drosophila melanogaster*, mice, and humans; only the C-terminal three regions are essential for assembly (amino acids [aa] 439 to 609), and the C-terminal four regions appear to be essential for DNA end binding (aa 254 to 609). Within the minimal active fragment of Ku86 necessary for subunit interaction (aa 449 to 732) and DNA binding (aa 334 to 732), a proline-rich region is the only defined motif.

There are two broad types of double-strand-break repair: homologous recombination and DNA end joining (37). *Saccharomyces cerevisiae* is capable of nonhomologous DNA end joining, but this process is much less efficient than the highly efficient homologous recombination in yeast cells. Perhaps because of this, none of the factors for DNA end joining have thus far been initially identified in *S. cerevisiae*. Ku and DNA-dependent protein kinase (DNA-PK) are two factors that have been identified on the basis of genetic complementation of mammalian cell lines deficient in their survival of ionizing (gamma) radiation and defective in the specialized form of double-strand-break repair called V(D)J recombination (7, 18, 32, 33, 38–41).

Though there are several proteins that are known to artifactually bind to double-stranded DNA (dsDNA) ends, the only one currently thought to physiologically bind DNA ends in a sequence-independent fashion is the dsDNA repair protein Ku. Ku is a heterodimer composed of polypeptides with apparent mobilities of 70 and 86 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The cDNAs for both subunits have been cloned, and the derived molecular masses are 70 and 83 kDa (30, 36, 46). Ku is conserved both functionally and structurally across a broad range of eukaryotes. *S. cerevisiae* and *Drosophila melanogaster* extracts contain DNA end binding activities which result from a heterodimer with a molecular weight similar to that of mammalian Ku. In both cases, the 70-kDa subunit was cloned and found to be homologous to mammalian Ku70 (4, 16).

DNA-PK is the only kinase thus far identified that is activated by DNA termini (8, 25). Recently, it has been suggested that Ku is the regulatory subunit of DNA-PK holoenzyme; on the basis of this hypothesis, the 470-kDa polypeptide alone is designated DNA-PKcs. On the basis of these studies, DNA-PK is proposed to be inactive in the absence of Ku and DNA termini. When DNA-PKcs assembles on DNA recognized by Ku in vitro, the kinase activity of DNA-PKcs is increased (7, 13, 20, 33). But in vivo, the substrates of DNA-PKcs remain to be defined. Ku is phosphorylated by DNA-PKcs in vitro (2). There are some data indicating that Ku has ATPase activity and acts as a helicase (42). There are approximately 5×10^5 Ku molecules and 0.5×10^5 to 1×10^5 DNA-PKcs molecules per nucleus (2).

Ku binds to dsDNA ends with high affinity and to single-stranded DNA with lower affinity (28). Binding to dsDNA ends includes binding to 5′ overhangs, 3′ overhangs, and blunt ends. Ku also binds to DNA nicks and to DNA termini ending in hairpin structures, perhaps by recognizing transitions from dsDNA to single-stranded DNA without specificity for any particular nucleotide sequence or end configuration (6, 15, 31). Moreover, DNA footprint analysis demonstrates that this protein selectively protects approximately 25 nucleotides at both the 5′ and 3′ termini from DNase I digestion. Once Ku binds to DNA ends, it is capable of translocating along the DNA in the absence of ATP, thereby permitting multiple molecules of Ku to load onto a single DNA fragment (12, 49). Recently, Ku has also been found to be capable of some sequence-specific binding to internal DNA sequences (not at DNA termini) (19).

Although Ku is well characterized for DNA binding activity, its cellular function has been unclear. Recent studies have indicated that Ku is important for double-strand-break repair (24, 26). V(D)J recombination is a specialized form of double-strand-break repair. Cell lines deficient in Ku show abnormalities in both double-strand-break repair and V(D)J recombination (32, 38, 40, 41).

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Several structural features with potential functional significance are apparent in the primary sequences of Ku70 and Ku86. For Ku70, the N terminus has an acidic domain (human amino acids [aa] 11 to 29) similar to the acidic transcriptional activation domain of some transcription factors. A second acidic region (aa 330 to 342) is not well conserved in *D. melanogaster* or *S. cerevisiae*. Two regions with some similarity to leucine zipper motifs are present, aa 215 to 243 and 483 to 518, but only the second one is conserved (35). Finally, a putative DNA binding domain (aa 585 to 604) has been mapped to the C-terminal region (designated a helix-turn-helix motif) on the basis of Southwestern (DNA-protein) analysis (9, 27, 31). In Ku86, the N terminus contains a leucine zipper motif (aa 138 to 165), and there is a proline-rich domain (aa 478 to 519) within the C-terminal region (45).

Questions about the assembly and structure of Ku and its interactions with DNA remain. The domains are predicted on the basis of the primary sequence. Are these truly functional? Which regions are the subunit interaction domains and which are the DNA binding domains within the Ku subunits? Do the Ku subunits bind to each other through a leucine zipper interaction? There are significant differences between the leucine zipper sequences found in Ku and those found in other DNA-binding proteins. How do the Ku subunits assemble on DNA ends? Does the association of the Ku70 and Ku86 subunits require DNA? The in vitro DNA binding studies of native Ku protein have not addressed these questions. Here, we have characterized the subunit interactions and the protein-DNA interaction domains of this unique DNA end-binding protein of eukaryotic cells.

MATERIALS AND METHODS

Yeast two-hybrid experimental design. The strategy has been fully described by Zervos et al. (48), but in brief, the method utilizes three plasmids (pEG202 [bait plasmid], pJG4-5 [prey or library plasmid], and pSH18-34 [reporter plasmid]) and a yeast strain, EGY48, that has an integrated *LEU2* gene with its upstream regulatory region replaced by LexA operators. The bait plasmid, pEG202, has an *ADH1* promoter upstream of the gene encoding a LexA protein, which can be fused to any bait protein. The LexA protein contains a DNA binding domain (for binding to the LexA operator region) and a LexA dimerization domain (for homodimer formation for LexA operator binding). This plasmid, like the other two, has a 2μm circle origin for yeast replication, the β-lactamase gene for prokaryotic selection, the ColE1 origin for prokaryotic replication, and a yeast metabolic selectable marker. For pEG202, this selectable marker is the *HIS3* gene.

The prey plasmid pJG4-5 has the *GAL1* promoter, which is galactose inducible and glucose repressible, upstream of B42 (which encodes an acidic RNA polymerase II activation domain) and fused to the gene encoding a prey protein or a library of such proteins. A simian virus 40 nuclear localization signal is located at the N-terminal portion of the B42 region. The yeast selectable marker on this plasmid is *TRP1*.

The reporter plasmid pSH18-34 carries multiple LexA operator regions upstream of the β-galactosidase gene. The β-galactosidase gene product produces a blue colony or suspension of cells for the spectrophotometric readout when X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is present on agar plates upon which *S. cerevisiae* is growing. The LexA operator is the DNA sequence that recruits the LexA-bait fusion protein. The stronger the interaction between the bait and prey proteins, the greater the localization of the B42 activator domain at the LexA operator, and hence the greater the initiation of RNA polymerase II from this region. This will increase the transcription of the β-galactosidase gene, resulting in a darker blue color of a colony or a higher spectrophotometric readout of β-galactosidase activity in a suspension of yeast cells.

A LexA operator was positioned upstream of both copies of the *LEU2* gene in the host yeast strain (EGY48). This operator functions identically to the LexA operator upstream of the β-galactosidase gene on pSH18-34. However, in this case, increased interaction between the bait and prey proteins will result in leucine prototrophy; otherwise, the yeast cells are auxotrophic for growth on media containing leucine.

Yeast two-hybrid strains and plasmid vectors. All strains were derived from *S. cerevisiae* EGY48 (*MAT***a** *trp1 ura3 his3 leu2*::pLexAop6-leu2).

After identification of the interaction between Ku70 or Ku86 and library members (in the prey vector, pJG4-5), a useful control involves switching Ku70 and Ku86 to the bait vector (pEG202) and carrying out the genetic test again with the bait and prey reversed. When convenient, this was done by direct cloning. Some of these truncated versions of Ku70 initially were identified by two-hybrid yeast selection with the Ku86 bait to screen an acidic fusion cDNA HeLa library cloned into the prey vector. The N-terminal Ku70 fragments, including the 48-, 44-, 41-, and 20-kDa fragments, were generated by *Eco*RI and *Xho*I liberation from the library prey vector, pJG4-5. These fragments were directly ligated into bait vectors that had been predigested with the same enzymes. The baits for the 81-, 45-, and 32-kDa domains of Ku86 were constructed by a similar procedure, but the bait for the 48-kDa domain of Ku70 was used to screen the HeLa library to identify these fragments.

In some cases, the swap between bait and prey vectors was carried out by PCR. To clone the cDNAs of full-length Ku70 and Ku86 or of the truncated versions of Ku70 (18 kDa [aa 290 to 450] and 50 kDa [aa 11 to 450]) or Ku86 (23 kDa [aa 1 to aa 210] and 62 kDa [aa 1 to 551]) into bait vectors to generate the DNA-binding constructs, a KlenTaqLA polymerase reaction mixture (from W. Barnes) was used to generate fragments suitable for ligation. The inserts contained a *Bam*HI site at the 5′ end and a stop codon and an *Xho*I site at the 3′ end for pEG202-Ku70 or the 50-kDa version of Ku70 and an *Eco*RI site at the 5′ end and a stop codon and an *Xho*I site at the 3′ end for pEG202-Ku86, the 23- and 62-kDa versions of Ku86, and the 18-kDa version of Ku70. When PCR was used, multiple clones were functionally analyzed. However, the error rate with KlenTaqLA polymerase mixture after 16 cycles is 1 in 20,000 (3). We anticipate that the large majority of our PCR products will have no mutations under these conditions, which are identical to those previously described (3). The functional comparison of multiple clones supports this inference.

The junctions of all constructs were sequenced by the Sanger dideoxy sequencing method.

LexA-bicoid is a control bait construct and was a gift from Roger Brent. This is an important and discriminating control because the LexA-bicoid fusion protein is known to enter the nucleus and to bind to the LexA operator recognition sites. Therefore, any interaction between a false prey and the LexA portion of the bait fusion protein would also be identified in the bicoid control as a false positive. In addition, a false-positive signal in the two-hybrid assay due to any effect of the prey fusion protein on any aspect of yeast physiology would also be detected as a false positive in the bicoid control test.

Two-hybrid library screening and protein-protein interaction assay. For determination of a potential interaction between truncated Ku70 and Ku86 proteins, a HeLa cDNA fusion library was screened by a procedure described previously (48). The plasmids for the two-fusion constructs (one fused with the LexA DNA binding domain and the other fused with the B42 transactivation domain) were cotransformed into the EGY48 strain, which had been pretransformed with a *lacZ* reporter plasmid, pSH18-34. Transformed yeast cells were selected on complete medium lacking uracil, His, and Trp and containing 2% glucose. After 3 to 4 days of incubation at 30°C, the colonies were picked and further analyzed with the leucine and the *lacZ* reporters as described previously (22).

Constructs for in vitro transcription and translation. The pET14b constructs containing full-length Ku70 were the result of ligation of *Bam*HI-*Xho*I blunt fragments from pEG202-Ku70 into pET14b digested with *Xho*I and blunted by Klenow fill-in synthesis with the Klenow fragment of *Escherichia coli* polymerase I. The full-length Ku86, the 81-, 45-, and 32-kDa Ku86 fragments, and the 48-, 44-, 41-, and 20-kDa Ku70 fragments were generated by liberation from the pJG4-5 library (prey) vector with *Eco*RI-*Xho*I followed by Klenow fill-in synthesis as described above for two-hybrid construction. All of these fragments were ligated into a pET14b vector digested with *Bam*HI and blunted by Klenow fill-in synthesis. The junction of each construct was sequenced. Multiple clones were analyzed to confirm that similar results were obtained.

In vitro transcription and translation of Ku proteins. One microgram of Ku70 or Ku86 cDNA in pET14b was used for in vitro transcription and translation under the control of the T7 promoter. The TnT rabbit reticulocyte lysate kit (Promega) was used according to the manufacturer's instructions. Full-length Ku70 with truncated Ku86 and full-length Ku86 with truncated Ku70 were cotranscribed and cotranslated.

Purification of in vitro-translated Ku products. In vitro-translated products with N-terminal His tags were used for Ni^{2+}-nitrilotriacetic acid-agarose purification in a batchwise fashion. In brief, a settled-bed volume of 100 μl of His-Bind resin was equilibrated with 1,000 μl of binding buffer which contains 20 mM Tris-HCl (pH 7.9), 5 mM imidazole, and 0.5 M NaCl. After this mixture was centrifuged for 1 to 2 min at 800 × *g* to remove binding buffer, 100 μl of translated reticulocyte lysate with [^{35}S]methionine label was mixed with Ni^{2+}-nitrilotriacetic acid resin in binding solution. Subsequently, the resin was washed three times with 200 μl of 20 mM Tris-HCl (pH 7.9)–20 mM imidazole–0.5 M NaCl. Finally, Ku proteins were eluted with 50 μl of elution buffer which contained 20 mM Tris-HCl (pH 7.0), 0.25 M imidazole, and 0.2 M NaCl. For the electrophoretic mobility shift assay, 15 μl of purified Ku was used. In parallel, 5 μl of [^{35}S]methionine-labeled products was resolved on an SDS–7% PAGE gel and was quantitated by phosphorimager analysis. The results indicated that similar amounts of protein were produced in all lanes.

Electrophoretic mobility shift assay. For preparation of a DNA probe, two complementary 79-nucleotide oligonucleotides were denatured and then annealed to generate a 71-bp fragment with *Bam*HI overhangs at each end (oligonucleotides ML113, GATCCTCTGAGGACACAGCCTTGTATTACTGTGCA

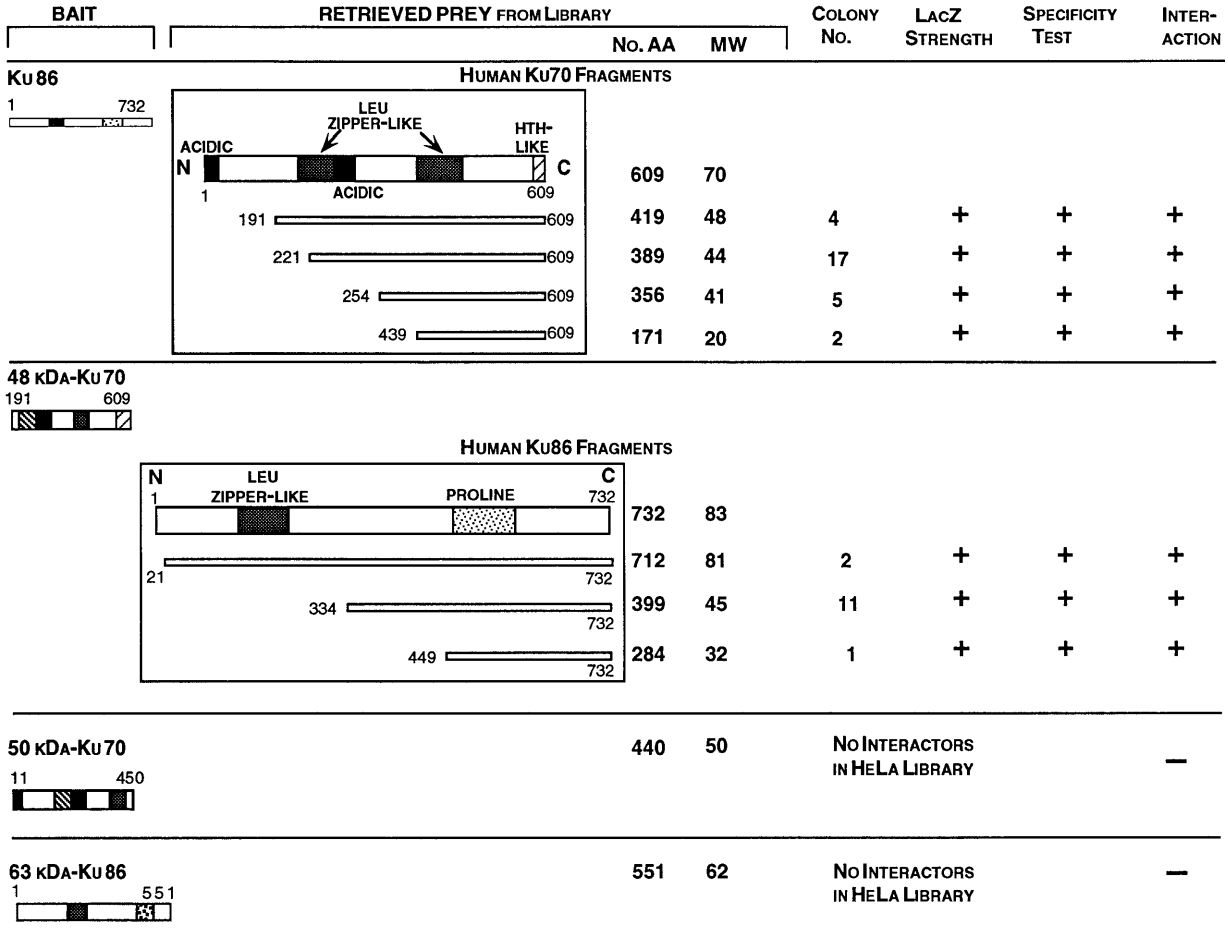

FIG. 1. Interactions detected by a yeast two-hybrid genetic assay. Fusion proteins of Ku86 or Ku70 with the LexA DNA binding domain were used (as baits) to search for library fusion members (as prey) that indicate interaction. The indicated number of colonies were detected under conditions described in Materials and Methods and Results. These were grouped according to similarity of restriction digestion pattern or sequence and then analyzed for specificity with the bicoid control (see Materials and Methods). AA, amino acid(s); HTH, helix-turn-helix; MW, molecular weight (in thousands).

AGACACACAATGAGCAAAAGTTACTGTGAGCTCAAACTAAAACC; ML114, GATCGGTTTTAGTTTGAGCTCACAGTAACTTTTGCTCATTGT GTGTCTTGCACAGTAATACAAGGCTGTGTACTCAGAG). The fragments were gel purified and end labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Ku protein binding activity to the DNA probe was measured by a gel electrophoretic mobility shift assay (34). Each reaction mixture of 20 μl contained 0.5 ng of radiolabelled probe, 15 μl of Ni^{2+}-purified Ku, and 1 μg of supercoiled competitor DNA (pJG4-5) in binding buffer (15 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9], 50 mM NaCl, 5 mM MgCl, 1 mM dithiothreitol, 0.5 mM EDTA, 1 mM Tris-HCl [pH 7.9], 5% glycerol). For all binding assays, the protein was added last and the reaction mixture was incubated at room temperature for 20 min. The reaction mixture was resolved by nondenaturing electrophoresis through a 6% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer. The gel was dried on Whatman 3M paper and exposed to Kodak film. Binding activity was quantitated by phosphorimager analysis. For exposure of the mobility shift gel, the ^{35}S radioactivity was blocked by an intensifying screen which did not block the ^{32}P radiation; this procedure indicated that similar amounts of protein were produced in all lanes.

RESULTS

Isolation of cDNAs encoding proteins interacting with full-length Ku86 and the C-terminal 48-kDa moiety of Ku70. To identify proteins that interact with full-length Ku86 or full-length Ku70, a HeLa cell library was screened by the yeast two-hybrid system (see Materials and Methods) of the Brent laboratory (17). As bait, full-length Ku86 or Ku70 was fused to the C terminus of the LexA DNA binding domain in pEG202. Strain EGY48 was cotransformed with the *lacZ* reporter plasmid pSH18-34 and pEG202-Ku86 or pEG202-Ku70. The transformed colonies were patched onto X-Gal indicator plates and plates lacking leucine to test whether the Ku86 and Ku70 baits activate transcription by themselves. Such determinations are a technical requirement when using the two-hybrid genetic selection because if a bait protein has an acidic domain of its own, it will give a false-positive readout even without binding to a prey fusion protein. Interestingly, the test results indicated that the LexA-Ku70 bait does have strong transcriptional activation activity for both the leucine reporter and the *lacZ* reporter but that the LexA-Ku86 bait does not. Ku70 has a strong acidic domain at its N terminus. This may be functionally important for roles that Ku70 may have in transcriptional regulation. In order to use Ku70 as a bait, it was necessary to cleave off the N-terminal 22-kDa portion of Ku70, leaving the 48-kDa C-terminal moiety.

S. cerevisiae harboring both the *lacZ* reporter plasmid and either the Ku86 or 48-kDa Ku70 bait plasmid was retransformed with the HeLa cell oligo(dT) cDNA library (constructed in pJG4-5 to make fusion proteins with the B42 transcription activation domain). When a cDNA encodes a protein that interacts with Ku86 or the 48-kDa Ku70 bait, the reporter strain is expected to grow in the absence of leucine and to produce β-galactosidase when on galactose-raffinose plates.

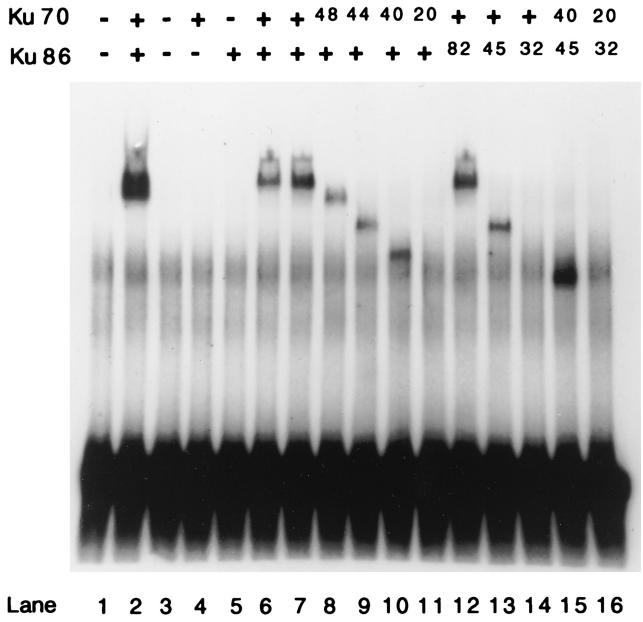

FIG. 2. DNA binding activities of Ku70 and Ku86 and their truncations. A 79-bp dsDNA oligonucleotide fragment (0.5 ng) was treated with kinase and used as a probe for DNA end binding activity (lane 1 shows the analysis of free probe without extract). As described in Materials and Methods, these assays are done in the presence of 1 μg of unlabeled supercoiled DNA competitor. For comparison purposes, human Ku70-Ku86 purified from HeLa cells (gift from M. Yaneva) demonstrates the mobility of the native Ku complex bound to the DNA (lane 2). Lanes 3 to 16 show the DNA binding of Ku70 and Ku86 derivatives generated by a reticulocyte-coupled transcription-translation system. Labeled [^{35}S]methionine was included in the cell-free synthesis to permit quantitation of the amount of specific protein produced; the amounts in all lanes were similar on the basis of phosphorimager quantitation (see Materials and Methods). Truncations from the cell-free translation were analyzed. Unless otherwise indicated, the cDNA constructs for the two subunits were transcribed and translated in the same reaction tube. Lanes are as follows, with percent shift in parentheses: 1, probe only (0); 2, native protein (24); 3, vector with no Ku70 or Ku86 coding region (0); 4, Ku70 (0); 5, Ku86 (0); 6, Ku70 plus Ku86, after independent translation (11.5); 7, Ku70 plus Ku86 (13.6); 8, 48-kDa Ku70 fragment plus Ku86 (4.7); 9, 44-kDa Ku70 fragment plus Ku86 (5.2); 10, 41-kDa Ku70 fragment plus Ku86 (4.7); 11, 20-kDa Ku70 fragment plus Ku86 (0); 12, Ku70 plus 81-kDa Ku86 fragment (13.4); 13, Ku70 plus 45-kDa Ku86 fragment (7.2); 14, Ku70 plus 32-kDa Ku86 fragment (0); 15, 41-kDa Ku70 fragment plus 45-kDa Ku86 fragment (9.2); 16, 20-kDa Ku70 fragment plus 32-kDa Ku86 fragment (0). +, full-length subunit was present; −, full-length subunit was absent.

The plasmids were extracted by a yeast miniprep. PCR was used to amplify the cDNA with primers derived from the vector pJG4-5. The resulting PCR products were digested with *Mbo*I to identify those that contain identical library inserts. At least one representative cDNA from each group was partially sequenced by the dideoxy method and tested for whether it specifically interacts with the original bait. More than one molecular clone yielded the same results.

About one million independent transformants of the HeLa cell library were searched with the Ku86 and 48-kDa Ku70 baits. For the Ku86 bait screen, of the 29 colonies that passed the leucine selection, 28 contained plasmids that induced transcription in a galactose-dependent fashion from both the *LEU2* and *lacZ* reporter constructs. Plasmids were rescued and used to retransform the reporter strain in combination with the original bait or with an unrelated gene fusion plasmid, such as the bicoid bait. All 28 passed the bicoid test and fell into four groups on the basis of *Mbo*I mapping of PCR products. The nucleotide sequences of all four groups showed a 100% match to the cDNA encoding the C terminus of human Ku70 (Fig. 1).

For the 48-kDa Ku70 bait screen, of the 124 colonies that passed through the initial selection, 16 activated the *lacZ* reporter upon induction of galactose-raffinose. Fourteen of the 16 colonies passed the bicoid test, and these were classified into three groups on the basis of *Mbo*I mapping of PCR products. The sequences of all of these were shown to be identical to the cDNA encoding the C terminus of human Ku86 (Fig. 1).

To further identify the protein interaction domains in both Ku70 and Ku86, the 44-, 41-, and 20-kDa C-terminal Ku70 baits were constructed and introduced into the EGY48 strain. This strain was retransformed with the 81-, 45-, and 32-kDa Ku86 prey individually to test whether they interact and to determine the minimal regions required for the proteins to interact with each other. The 20-kDa portion of Ku70 (171 aa of the C terminus) specifically interacts with the 32-kDa portion of Ku86 (283 aa of the C terminus) in the yeast two-hybrid system. Neither the 45- nor the 32-kDa portion of Ku86 includes the leucine zipper-like domains present in the full-length Ku86. The results show that the association of the Ku subunits is not mediated by a leucine zipper motif in either of the polypeptides. The truncated versions of the Ku subunits clearly form complexes within yeast cells on the basis of the two-hybrid genetic analysis, which included the bait-prey interchange (see Materials and Methods) and bicoid controls.

DNA binding activity for truncated Ku complex. The question of whether these complexes are functional in DNA binding remained. To determine whether these complexes bind to DNA and to define the DNA binding domains within the Ku70 and Ku86 subunits, the in vitro translation products described above (see Materials and Methods) were tested for binding to DNA in a mobility shift assay. In initial studies, we found that reticulocyte lysates contained a factor which nonspecifically binds to linear DNA. To eliminate this background, the in vitro-translated Ku products were purified with Ni^{2+}-nitrilotriacetic acid resin in a batchwise fashion. Ku70 and Ku86 were either translated alone or cotranslated with Ku86 and Ku70, respectively, in the presence of [^{35}S]methionine. The vector without an insert was used as a control for in vitro translation. Generally, 15 μl of purified Ku protein product yielded a satisfactory result for the mobility shift. In parallel, 5 μl of [^{35}S] methionine-labeled products were resolved on an SDS–7% PAGE gel. The density of each band corresponding to the truncated version of Ku was quantitated by phosphorimager analysis and normalized for the number of methionine residues in each Ku fragment. The calibrated density was used in this manner to determine the relative protein amount.

The results demonstrate that individual full-length Ku subunits do not interact with DNA to produce a Ku-DNA complex band (Fig. 2, lanes 4 and 5). But when individually translated full-length Ku70 and Ku86 are mixed and tested for a mobility shift (Fig. 2, lane 6), there is reconstitution of the Ku-DNA complex just as in the cotranslation of both subunits (Fig. 2, lane 7). This also means that cotranslation of Ku70 and Ku86 is not required for formation of the heterodimer.

For the truncated forms, we found that the C-terminal 41-kDa domain of Ku70 and the C-terminal 45-kDa domain of Ku86 are sufficient to interact with each other and retain nearly wild-type DNA binding activity (Fig. 2, lane 15). Shorter versions of Ku70 (C-terminal 20-kDa domain) and Ku86 (C-terminal 32-kDa domain) are able to assemble into a heterodimeric complex (on the basis of the yeast two-hybrid system data), but they no longer have DNA binding activity (Fig. 2, lanes 11, 14, and 16).

The truncations of Ku70 and Ku86 that do heterodimerize and that bind to DNA termini have a relative binding affinity that is similar to that of full-length Ku. On the basis of densi-

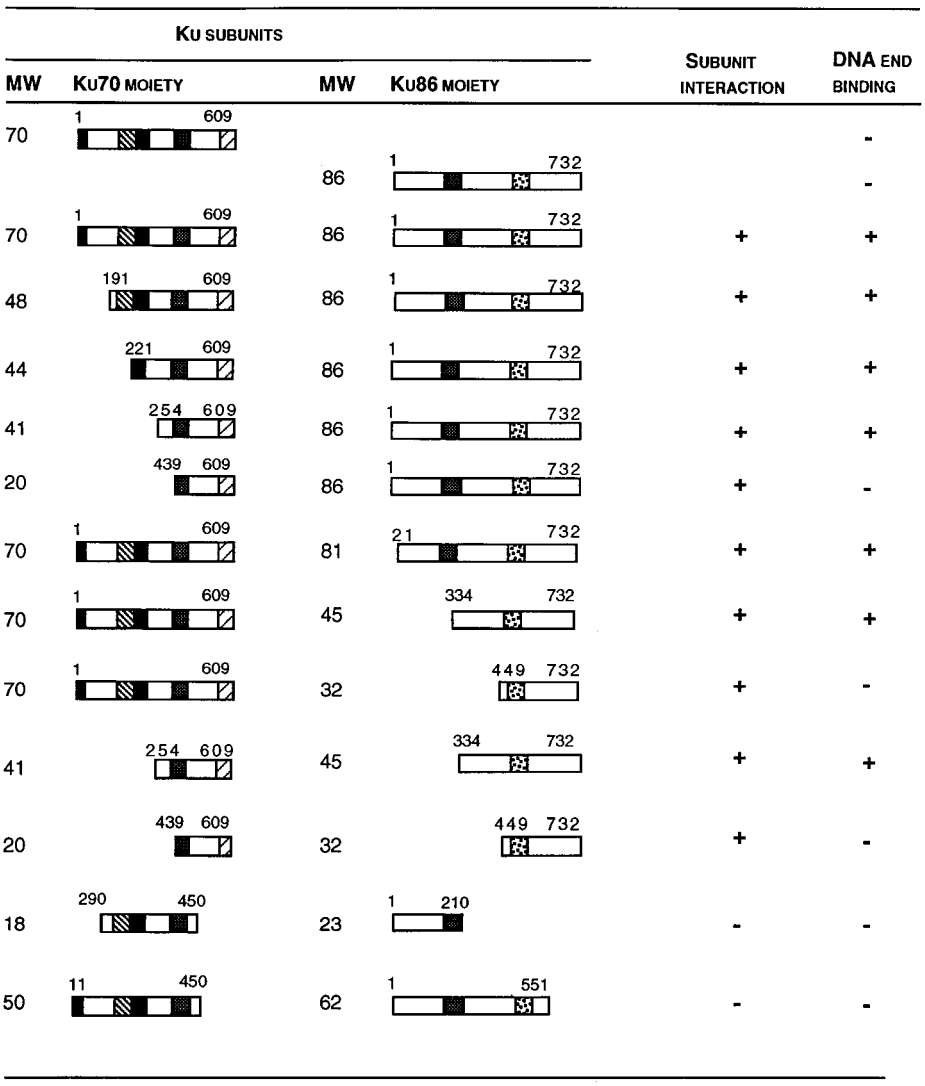

FIG. 3. Interaction and DNA binding regions of Ku70 and Ku86. Ku70, Ku86, and their derivative fragments were tested individually or together for two-hybrid interaction (subunit interaction) and for DNA end binding by the electrophoretic mobility shift assay (see the Fig. 4 legend). Gel mobility shift data for the last two pairs of Ku subunits are not shown in Fig. 2 but have been seen on other gels (data not shown). MW, molecular weight (in thousands).

tometric analysis of the mobility shifts, the extent of the shifts for the truncated Ku polypeptides is within a factor of 2 to 3 of that of full-length Ku70 and Ku86.

DISCUSSION

The assembly and interaction of Ku and DNA-PK at the sites of double-strand breaks are areas of intense interest. Among the questions that are of central importance are those concerning the assembly and DNA binding of Ku at the DNA ends: which portions of Ku are important for subunit assembly, and which portions are important for DNA end binding?

Individual Ku subunits fail to bind DNA ends. Our data show that only the assembled Ku70-Ku86 complex has DNA end binding activity under native conditions (Fig. 2). Individually translated full-length Ku70 and Ku86 and individually translated truncated Ku subunits, including the C-terminal leucine zipper-like motif and the basic region helix-turn-helix motif, all lack DNA binding activity when tested in the absence of the heterodimeric partner. This indicates that dimerization of Ku subunits is required for DNA binding.

Previous work indicated that Ku70 is likely to be the active DNA end-binding member of the dimer, partly on the basis of UV cross-linking data (49). Additional studies raised the possibility that Ku70 might carry out this DNA end binding function in the absence of Ku86 (1, 11, 43, 44). Among these was a Southwestern analysis in which Ku70 and Ku86 were separated by SDS-PAGE and renatured on nitrocellulose membranes (29).

The present study and that of Griffith et al. (21) using the mobility shift assay indicate that Ku70 does not bind by itself to DNA, whereas the same Ku70 does bind to DNA when Ku86 is present. The observations that Ku70 may interact with DNA in the absence of Ku86 may be explained in part by exposure of nonphysiologic (nonspecific) binding regions during the denaturation step of the Southwestern procedures. In addition, these studies were done with fusion proteins that may generate nonphysiologic binding regions upon renaturation. One can

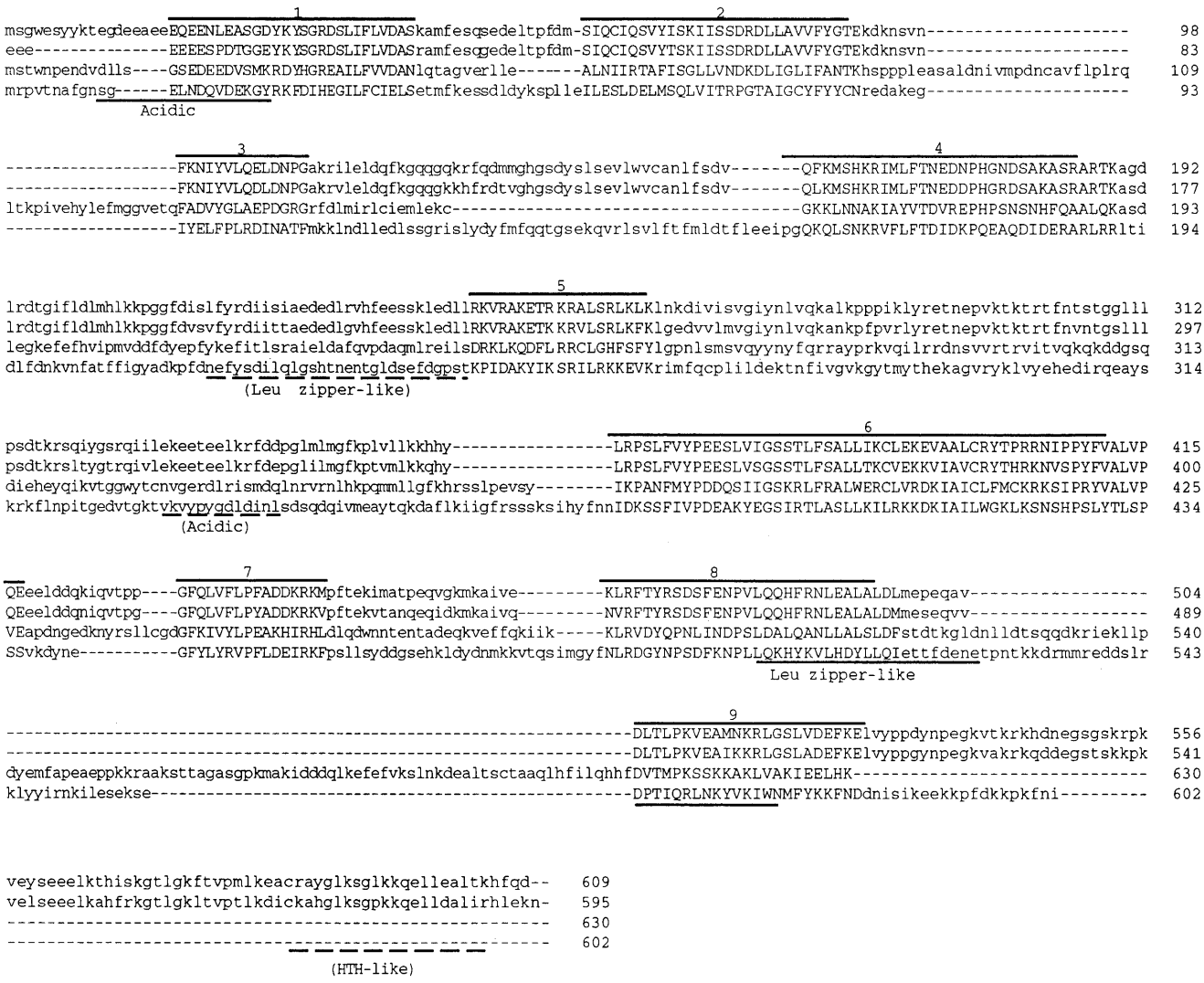

FIG. 4. Conserved sequence domains in Ku70. The human (top line), murine (second line), *D. melanogaster* (third line), and *S. cerevisiae* (bottom line) Ku70 proteins were aligned and analyzed for conserved sequences by using the MACAW program of the National Center for Biotechnology Information. Nine regions of conserved homology with the highest statistical significance between human, mouse, *D. melanogaster*, and yeast Ku70 were identified, and these are in capital letters (conserved domain numbers 1 through 9 are indicated above the corresponding segments of sequences). All had statistical sequence probabilities (of occurrence by chance) of 2×10^{-8} or less except two; these were region 3 ($P < 0.004$) and region 5 ($P < 0.1$). Acidic (Asp-Glu) regions, leucine zipper domains, and helix-turn-helix domains suggested previously by others are designated below each line of sequence. Of these, those that are highly conserved and that are within the functional regions for dimerization and DNA binding are indicated by solid lines. Those that are not conserved across the four species and that are not within the conserved regions are indicated by dashed lines. HTH, helix-turn-helix.

imagine that such nonphysiologic regions could form within either moiety of the fusion protein; but at the interface of the two moieties, entirely novel domains may form upon renaturation. Additional studies (43, 44) used vaccinia virus overexpression of human Ku70 and Ku86 in rabbit cells. Detection of Ku70 and Ku86 relied on Western blots (immunoblots). The experiments were done with crude extracts from cells infected with Ku70 alone, Ku86 alone, both, or neither. This approach assumes that there is no mixing between the overexpressed human Ku70 and the rabbit cell Ku86, but mixing may not be readily detected with anti-human Ku86 antibodies. What is hoped to be Ku70 by itself may actually be human Ku70 heterodimerized with rabbit Ku86.

Minimal regions of Ku70 and Ku86 necessary for subunit interaction. The deletion analysis that we have done defines domains which are and are not necessary for subunit interaction (Fig. 3). The C-terminal 20-kDa domain of Ku70 interacts with the 32-, 45-, 81-, and 86-kDa portions of Ku86. On this basis, we conclude that conserved-sequence regions 7 (last half), 8, and 9 (in the last 171 aa) are needed for heterodimer assembly and domains 1 through 6 are not (Fig. 4). When conserved-sequence regions 8 and 9 are deleted, as they are in the 50-kDa version of Ku70, no interactions are detected (Fig. 1). Between regions 8 and 9, there is a weak leucine zipper motif. The weak helix-turn-helix motif in the last 25 aa of the human Ku70 is not present in the *S. cerevisiae* or *D. melanogaster* Ku70; hence, we have not assigned this sequence a region designation.

The C-terminal 32-kDa portion of Ku86 interacts with the 20-, 41-, 44-, and 48-kDa portions of Ku70 as well as the full-length version. On this basis, we infer that the weak leucine zipper motif between aa 138 and 165 in Ku86 is dispensable for

subunit interaction. The leucine zipper motif in domains 8 and 9 of Ku70 is unlikely to be the site of functional interaction with Ku86 because there is no corresponding leucine zipper within the minimal region of Ku86 with which it can interact. The proline-rich domain (aa 478 to 519) is located within the 32-kDa minimal region of Ku86 required for subunit interaction. When the C-terminal 181 aa (aa 552 to 732) are deleted from Ku86, the truncated version fails to interact (last line of Fig. 1), confirming the importance of the C-terminal 284-aa 32-kDa portion of Ku86 for subunit interaction (aa 449 to 732).

Minimal regions of Ku70 and Ku86 required for DNA end binding. Within Ku70, the region between aa 1 and 254 is dispensable for DNA end binding. Unlike the case of subunit interaction, however, the region from aa 254 to 439 is essential for DNA end binding. The region from aa 254 to 439 is within conserved region 5 and extends halfway through domain 7. Therefore, the segment from region 5 to the C-terminal portion (region 9) appears to be important in DNA end binding.

For Ku86, a comparison of the 32- and 45-kDa portions is informative. The 45-kDa fragment of Ku86 interacts with Ku70, and the heterodimer binds DNA ends. Though the 32-kDa fragment of Ku86 assembles with Ku70, the heterodimer fails to bind DNA. Therefore, the region from aa 334 to the C terminus (aa 732) is essential to achieve both assembly and DNA binding, even though the shorter region (from aa 449 to 732) is sufficient for assembly alone.

Understanding the impact of Ku mutations on double-strand-break repair. Determination of the regions of Ku70 and Ku86 dispensable for dimerization and for DNA end binding permits examination of Ku mutations in light of this additional information. We and others have characterized the V(D)J recombination and ionizing-radiation repair capabilities of mammalian cell mutants with apparent defects in the Ku heterodimer (5, 10, 23, 32, 41). Recently, the precise details of two mutations in Ku86 were defined (14). Both are in-frame internal deletions. One of these mutations, in XR-V9B, deletes the region from aa 267 to 350. The other mutation, in XR-V15B, deletes the region from aa 372 to 417.

Our analysis here includes two deletions that may contribute to understanding the effects of the above-described deletions. Both the 45-kDa (deletion of aa 1 to 333) and the 32-kDa (deletion of aa 1 to 448) portions of Ku86 heterodimerize with full-length Ku70. However, only the 45-kDa portion binds to DNA ends; the 32-kDa portion does not (when complexed with Ku70).

Previously, we found that the V(D)J recombination defect of XR-V9B (32) was less severe than that which we and others have subsequently found to be the case for XR-V15B (14). In addition, the radiation sensitivity of XR-V9B is less severe than that of XR-V15B (47). It is interesting that though both mutants are affected, the more severely affected mutant, XR-V15B, has a cDNA deletion (aa 372 to 417) that includes a large portion of the region between aa 334 and 448 of Ku86 that we determined to be essential for DNA binding. In contrast, the large majority of the cDNA deletion in XR-V9B is in the portion dispensable for DNA binding.

Concluding remarks. We have analyzed regions of Ku70 and Ku86 important for Ku heterodimer assembly and DNA end binding. In this two-hybrid analysis, we did not detect any interaction with other proteins. The lack of detection of DNA-PKcs may be due to the fact that its mRNA is 13.5 kb. Hence, detection of interactions is likely to be constrained in libraries priming in the poly(A) tail. We are currently conducting a similar analysis using segments of DNA-PK as the bait to determine if some of these interact with Ku.

We have not addressed the potential role of Ku as a sequence-specific transcription factor, an issue that has been raised most recently in connection with a glucocorticoid-responsive promoter (19). Ku may have a role both as a DNA end-binding protein and as a sequence-specific transcription factor, and the regions of Ku relevant for these roles may differ. Of potential significance for Ku's role in transcription is our finding that full-length Ku70 strongly activates transcription in both the leucine and *lacZ* reporter assays, while the C-terminal 48-kDa Ku70 bait does not. This bait has the N-terminal 190 aa deleted, and it is this N-terminal segment that contains the conserved acidic domain. Our observations suggest that if Ku has a role in transcription, the N-terminal acidic domain of Ku70 may be responsible for transcriptional activation.

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