A Model for Ku Heterodimer Assembly and Interaction with DNA

IMPLICATIONS FOR THE FUNCTION OF Ku ANTIGEN*

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Ku autoantigen, a heterodimer of 70- and 80-kDa subunits, is a DNA end-binding factor critical for DNA repair. Two domains of p70 mediate DNA binding, one on the C-terminal and one on the N-terminal portion. The latter must dimerize with p80 in order to bind DNA, whereas the former is p80-independent. Both must be intact for end binding activity in gel shift assays. To evaluate the role of p80 in DNA binding, deletion mutants were co-expressed with full-length p70 using recombinant baculoviruses. We show by several criteria that amino acids 371–510 of p80 interact with p70. Both of the p70 dimerization domains bind to the same region of p80, but apparently to separate sites within that region. In DNA immunoprecipitation assays, amino acids 179–510 of p80 were required for p80-dependent DNA binding of p70, whereas in gel shift assays, amino acids 179–732 were necessary. Interestingly, both the p80-dependent and the p80-independent DNA binding sites preferentially bound to DNA ends, suggesting a model in which a single Ku heterodimer may juxtapose two broken DNA ends physically, facilitating their rejoining by DNA ligases.

Ku antigen was identified and characterized using autoantibodies from the sera of patients with systemic autoimmune diseases (reviewed in Ref. 1). Later, it was shown to be associated with a DNA-dependent protein kinase that phosphorylates chromatin-bound proteins *in vitro* (2) and is involved in double-stranded (ds)[1] DNA break repair (DSBR), V(D)J recombination, and isotype switching (3–5), as well as telomeric length maintenance and silencing (6). Ku is a heterodimer of 70-kDa (p70) and ~80-kDa (p80) subunits that binds dsDNA ends (1, 7). Sequence-specific DNA binding also has been reported (1, 8). Defining the contribution of p70 and p80 to DNA binding is of interest because of the dual sequence- and end-specific binding of Ku and because of uncertainty as to its precise role in DNA repair. In DNA immunoprecipitation and Southwestern blot assays, p70 binds DNA in the absence of p80 (7, 9, 10), but in gel shift assays, only the dimer can bind (11, 12). Although p80 alone does not bind to DNA, DSBR mutants

in the XRCC5 complementation group have defects in the p80 gene (13–15).

Defining the mechanism of p70-p80 dimerization may help to explain the role of p80 in sequence-specific DNA binding, DNA end binding, and/or DSBR. The p70 subunit contains two p80 interaction domains, amino acids 1–115 and 430–482, respectively, as well as two regions involved in DNA binding, each partially overlapping one of the interaction domains (16). A p80-independent DNA binding site is located on the C terminus (amino acids 536–609) (16, 17), whereas the N-terminal region must bind p80 in order to bind DNA. The goal of this study was to define the dimerization-dependent DNA binding site. Using p70 and p80 mutants, a large central domain of p80 involved in both dimerization and DNA binding was identified. This site, like the p80-independent DNA binding site, preferentially recognized DNA ends, suggesting that a single Ku heterodimer may bind two DNA ends.

EXPERIMENTAL PROCEDURES

Cells and Viruses—K562 (human erythroleukemia) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin. The Sf9 (*Spodoptera frugiperda* ovary) cell line was obtained from the ATCC, and maintained at 27 °C in Grace's insect tissue culture medium supplemented with 3.3 g/liter TC yeastolate, 3.3 g/liter lactalbumin hydrolyzate, 10% fetal bovine serum, and penicillin/streptomycin.

The human p70 and p80 Ku cDNAs were subcloned into a modified baculovirus transfer vector pVLB4-mp53 and co-transfected with linearized wild-type baculovirus into Sf9 cells as described (16). The recombinant baculovirus vectors direct the expression of Ku proteins fused to an N-terminal polyhistidine sequence and a enterokinase cleavage site. The recombinant baculoviruses expressing the full-length p80 or p70 are designated p80-bv and p70-bv respectively.

Recombinant baculoviruses expressing mutant human p80 were constructed in the same manner. Truncated p80 cDNAs were amplified by polymerase chain reaction using primer pairs listed in Table I, and subcloned into pVLB4-mp53.

Recombinant Protein Expression—Sf9 cells were infected with one or more recombinant baculoviruses as described (16). Seventy-two hours after infection, recombinant Ku proteins were identified in cell lysates by SDS-PAGE and immunoblotting using human autoimmune serum and/or specific monoclonal antibodies (mAbs).

mAbs—mAbs specific for the human Ku antigen were as described previously (16). Their isotypes and specificities are as follows: 162, IgG2a anti-p70/p80 dimer; S10B1, IgG1 anti- p80 (amino acids 179–371); 111, IgG1 anti-p80 (amino acids 610–705); N3H10, IgG2b anti-p70 (amino acids 529–541). An IgG2a murine mAb specific for the polyhistidine tag (HIS-1) was from Sigma.

Immunoblotting and Immunoprecipitation—Immunoblot analysis of the recombinant p70 and p80 proteins from Sf9 cells or Ku proteins from K562 cells was performed using murine mAbs (1:1000 dilution) (16). Radiolabeling of baculovirus-infected Sf9 cells with [35S]methionine/cysteine and immunoprecipitation onto protein A-Sepharose beads were carried out as described (16). The beads were washed with 1.5 M NaCl NET-Nonidet P-40 buffer (1.5 M NaCl, 50 mM Tris, pH 7.5, 0.3% Nonidet P-40, 2 mM EDTA) and once with NET buffer (0.15 M NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA). Immunoprecipitated proteins

[1] The abbreviations used are: ds, double-stranded; DSBR, double strand break repair; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; XRCC, x-ray cross-complementing.

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TABLE I

Primers used to amplify Ku cDNAs for subcloning into baculovirus transfer vectors

Constructs	5′ Primer	3′ Primer
p80.1–178	5′-GCGGATCCATGGTGCGGTCGGGGAATAAG-3′	5′-GCGAATTCTCATCTGTCCCCACTTCCATC-3′
p80.371–732	5′-GCGGATCCGAGGCAGCTGCAGTTGCAC-3′	5′-GCGAATTCCGACCTATATCATGTCC-3′
p80.179–732	5′-GCGGATCCGGAGATGGCCCCTTTCGC-3′	5′-GCGAATTCCGACCTATATCATGTCC-3′
p80.179–654	5′-GCGGATCCGGAGATGGCCCCTTTCGC-3′	5′-GCGAATTCTCAGCGCTGCTCTTCTGAAAAC-3′
p80.179–510	5′-GCGGATCCGGAGATGGCCCCTTTCGC-3′	5′-GCGAATTCTCACTGCTGAATTGGGGGTAG-3′
p80.179–400	5′-GCGGATCCGGAGATGGCCCCTTTCGC-3′	5′-GCGAATTCTCATCTTTTGTCATAAGC-3′
p80.371–654	5′-GCGGATCCGAGGCAGCTGCAGTTGCAC-3′	5′-GCGAATTCTCAGCGCTGCTCTTCTGAAAAC-3′
p80.371–510	5′-GCGGATCCGAGGCAGCTGCAGTTGCAC-3′	5′-GCGAATTCTCACTGCTGAATTGGGGGTAG-3′
p80.400–732	5′-GCGGATCCAGAGCTAATCCTCAAGTC-3′	5′-GCGAATTCCGACCTATATCATGTCC-3′
p80.400–654	5′-GCGGATCCAGAGCTAATCCTCAAGTC-3′	5′-GCGAATTCTCAGCGCTGCTCTTCTGAAAAC-3′
p80.400–510	5′-GCGGATCCAGAGCTAATCCTCAAGTC-3′	5′-GCGAATTCTCACTGCTGAATTGGGGGTAG-3′
p80.510–654	5′-GCGGATCCCAGCATATTTGGAATATGC-3′	5′-GCGAATTCTCAGCGCTGCTCTTCTGAAAAC-3′
p80.654–732	5′-GCGGATCCCGCTTTAACAACTTCCTG-3′	5′-GCGAATTCCGACCTATATCATGTCC-3′

were analyzed by SDS-PAGE.

Limited Protease Digestion—Ku heterodimer from K562 cells was affinity-purified onto mAb 162-coated protein A-Sepharose beads and digested with proteases (16). Protease V8 (Boehringer Mannheim), trypsin-treated tosylphenylalanyl chloromethyl ketone (Millipore Corp., Freehold, NJ), or chymotrypsin (Worthington Biochemical Corp., Freehold, NJ) were added to the beads at 2–200 μg/ml in 10 mM Tris-HCl, pH 7.5, and 2.5 mM CaCl$_2$ for 30 min at 22 °C. The beads were washed, and p80 fragments remaining associated with the beads were identified by SDS-PAGE and immunoblotting using S10B1.

Cell Extracts—Sf9 or K562 cells were washed briefly with hypotonic buffer (10 mM HEPES, pH 7.3, 25 mM KCl, 10 mM NaCl, 1 mM MgCl$_2$, 0.1 mM EDTA), and pellets were resuspended at 2×10^8 cells/ml in the same buffer supplemented with 0.5 mM dithiothreitol, 1 mM phenyl-methylsulfonyl fluoride, and aprotinin (0.3 trypsin inhibitor units/ml). Pellets were lysed by three freeze-thaw cycles and adjusted to 0.5 M KCl and 10 mM MgCl$_2$. After centrifuging, the supernatant was diluted to 0.1 M KCl and stored at −80 °C.

DNA Binding Assays—A 564-base pair linear double-stranded DNA fragment from bacteriophage λ was radiolabeled with [^{32}P]dATP, and its binding to affinity-purified Ku antigen was measured by DNA im-munoprecipitation assay (16). Briefly, cell extract was immunoprecipi-tated on protein A-Sepharose beads with 3 μl of N3H10, 111, or HIS-1 ascitic fluid. Equal amounts of recombinant Ku proteins were bound to the beads, as determined by SDS-PAGE with Coomassie Blue staining. Beads were washed with 1.5 M NaCl NET-Nonidet P-40 buffer, followed by the same buffer containing 50 mM NaCl. Radiolabeled DNA (25 ng) was added for 1 h, and the beads were washed again with NET-Nonidet P-40. Bound DNA was recovered by proteinase K digestion, and an aliquot of the supernatant was used for scintillation counting.

For gel mobility shift assays, radiolabeled DNA was purified using a Geneclean III kit (Bio 101, Vista, CA) and then incubated with cell extracts in 20 μl of binding buffer (10 mM Tris, pH 7.5, 1 mM dithio-threitol, 5 mM EDTA, 10% glycerol, 150 mM NaCl, 2 μg of closed circular ϕX174 Rf I DNA) at 22 °C for 30 min (16). Samples were analyzed by electrophoresis in a 4% polyacrylamide gel, followed by autoradiogra-phy. For supershift experiments, purified mAbs (~1 μg) were added to the binding mixture 5 min after adding probe and incubated for an additional 30 min at 22 °C before electrophoresis.

RESULTS

Two regions of the p70 interact with p80. Binding of p80 to one of these interaction domains contributes to the DNA bind-ing activity of Ku. In the present study, we investigated the mechanism of p80-p70 dimerization and its role in DNA binding.

Dimerization Domain of p80—The leucine zipper-like se-quence of p70 is not required for dimerization with p80 (16). The role of the leucine zipper-like sequence near the N termi-nus of p80 (18) in interactions with p70 was investigated by co-expressing a panel of p80 deletion mutants (Fig. 1A) with full-length p70 in Sf9 cells using recombinant baculoviruses. Recombinant protein expression was verified by immunopre-cipitation using the polyhistidine-specific mAb HIS-1 (Fig. 1, B and C, HIS). To detect heterodimer formation, the p80 mutants were co-immunoprecipitated with anti-p70 mAb N3H10 (Fig. 1, B and C, N3H10). Mutant proteins 179–732, 179–654, 179–

510, 371–654, and 371–510 were co-immunoprecipitated by N3H10, but 179–400 was not (Fig. 1B), suggesting that an interaction domain is located in the central portion of p80 (amino acids 371–510), and that the leucine zipper-like region (amino acids 146–178) is not essential for dimerization with p70. Even though 179–654 and 179–510 dimerized with p70 less efficiently than 179–732, their interactions were visual-ized readily by co-immunoprecipitation assay. Since 179–510 interacted with p70, whereas 179–400 did not, amino acids 400–510 may be crucial for dimerization with p70. However, because 179–400 was expressed less efficiently than the other constructs, it is difficult to completely exclude the possibility that some dimerization might occur with this construct.

Mutant 1–178 overlaps the leucine zipper-like sequence, but was not co-immunoprecipitated by N3H10 (Fig. 1C), indicating that the leucine zipper-like sequence of p80, like that of p70, is not required for dimerization. Mutant 400–732 was co-immu-noprecipitated by N3H10 but not 400–510, 510–654, or 654–732 (Fig. 1C). Thus, even though amino acids 400–510 are crucial for p70-p80 dimerization, this region by itself is insuf-ficient. Additional amino acids either N-terminal (*e.g.* mutant 371–510) or C-terminal (*e.g.* mutant 400–732) to the "core" domain (amino acids 400–510) are necessary for binding to p70.

Contribution of p80 to Dimerization-specific 162 Epitope—mAb 162 recognizes an epitope created by dimerization of p70 with p80 and prevents their dissociation in the presence of 1.5 M NaCl and detergent (19). The 162 binding site on p70 (amino acids 430–542) overlaps the C-terminal interaction domain of p70 (amino acids 430–482). The relationship of the 162 binding site to the interaction domain of p80 was investigated (Fig. 2, A–C). Expression of p70 and p80 deletion mutants was verified by HIS-1 antibody. mAb 162 immunoprecipitated full-length p70 when co-expressed with p80 mutant 179–732, but not 371–732, 179–371, or 1–178 (Fig. 2B), suggesting that the C-terminal half of p80 contributes to formation of the epitope. mAb 162 also immunoprecipitated p70 when co-expressed with p80 179–654 or 179–510, but not 179–400 (Fig. 2C), but it did not immunoprecipitate p70 or any of the p80 deletion mutants when expressed alone in Sf9 cells (20). These data confirm the dimerization studies illustrated in Fig. 1B. We conclude that amino acids 179–510 of p80 contribute to formation of the 162 epitope, and that this region overlaps the dimerization domain of p80.

To further define the minimal 162 epitope, p70 (amino acids 430–542) (16) was co-expressed with p80 mutants. mAb 162 immunoprecipitated p70 (430–542) when co-expressed with p80 (179–654 or 179–510), but not p80 (179–400) (data not shown), indicating that the 162 epitope consists of p70 (430–542) plus p80 (179–510).

Localization of the Dimerization Site by Limited Proteoly-

Ku Heterodimer Assembly
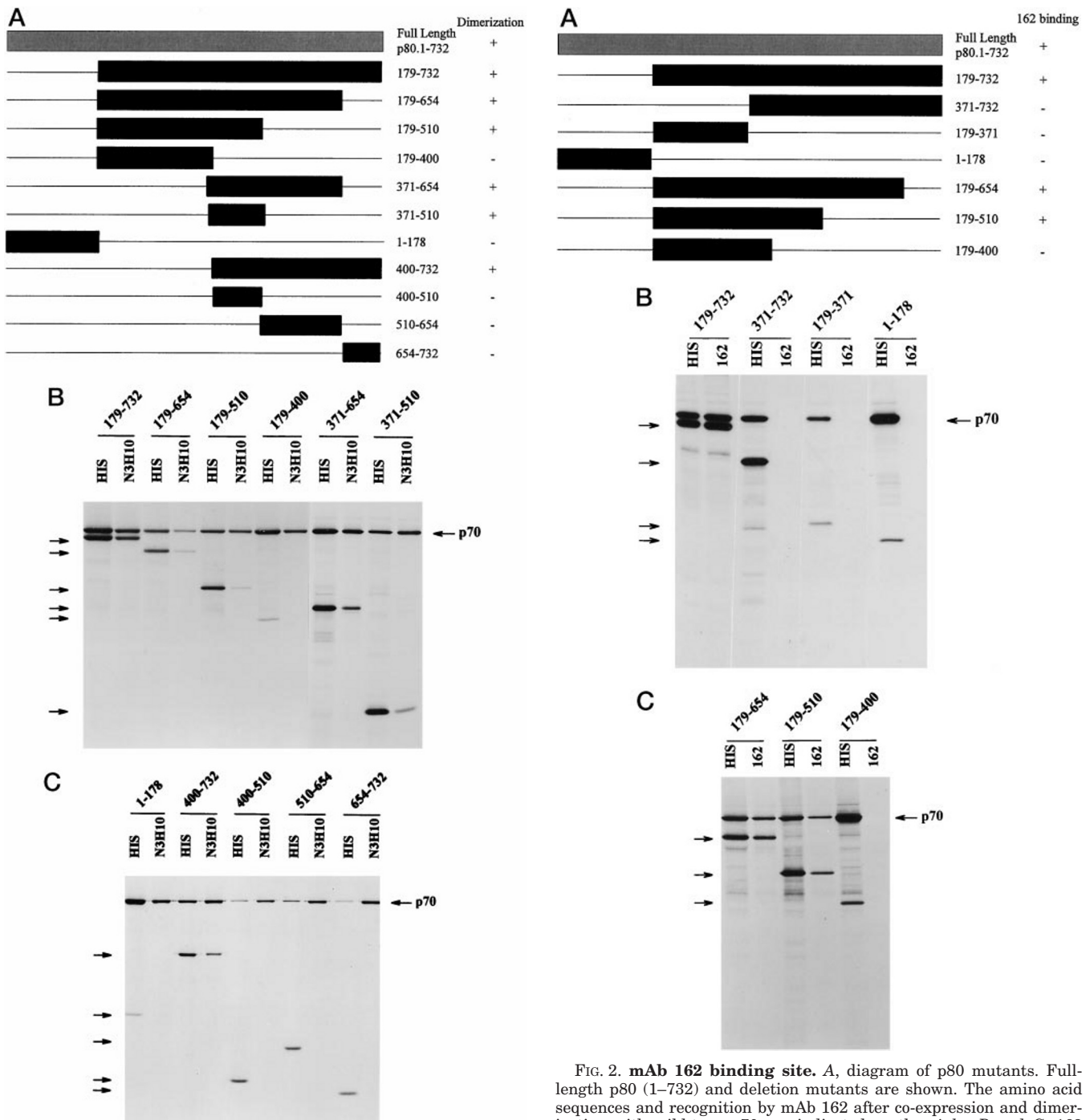
FIG. 1. **Dimerization domain of p80.** *A*, diagram of mutant p80 proteins. Wild-type full-length (1–732) p80 and deletion mutants expressed in Sf9 cells infected with recombinant baculoviruses are shown. The amino acid sequences and ability to dimerize with p70 of each construct are indicated on the *right*. *B* and *C*, co-immunoprecipitation of p70 and p80 mutants. Sf9 cells co-infected with p70-bv and recombinant baculoviruses expressing p80 mutants were radiolabeled, and cell lysates immunoprecipitated with anti-polyhistidine mAb (*HIS*) or anti-p70 mAb (*N3H10*) followed by SDS-PAGE and autoradiography. Positions of p70 and the individual p80 mutants are indicated by arrows.

FIG. 2. **mAb 162 binding site.** *A*, diagram of p80 mutants. Full-length p80 (1–732) and deletion mutants are shown. The amino acid sequences and recognition by mAb 162 after co-expression and dimerization with wild-type p70 are indicated on the *right*. *B* and *C*, 162 immunoprecipitation. Sf9 cells co-infected with p70-bv and recombinant baculoviruses expressing p80 mutants 179–732, 371–732, 179–371, or 1–178 (*B*), or 179–654, 179–510, or 179–400 (*C*), respectively, were radiolabeled, and cell lysates immunoprecipitated with anti-polyhistidine mAb (*HIS*) or anti-p70/p80 dimer-specific mAb 162, followed by SDS-PAGE and autoradiography. Positions of p70 and individual p80 mutants are indicated by *arrows*.

sis—To confirm the co-immunoprecipitation data, dimerization of p70 and p80 was studied in the human K562 cell line. Binding of mAb 162 protects the p70 interaction domain from proteolysis (16), suggesting that the corresponding region of p80 may be protected, as well. Ku heterodimer affinity-purified on mAb 162 was treated with proteases, and p80 fragments retained on 162 beads after protease treatment were analyzed

by Western blotting using mAb S10B1 (Fig. 3). The smallest bead-associated p80 fragment after chymotrypsin digestion was a weak, but consistently visualized, band migrating at ~22 kDa. Western blot analysis using mAb 111 (anti-p80, 610–705) failed to detect fragments smaller than 60 kDa (data not shown). Since S10B1 recognizes amino acids 179–371 of p80, the protease studies support the immunoprecipitation data, indicating that a dimerization site is located in the central portion of p80.

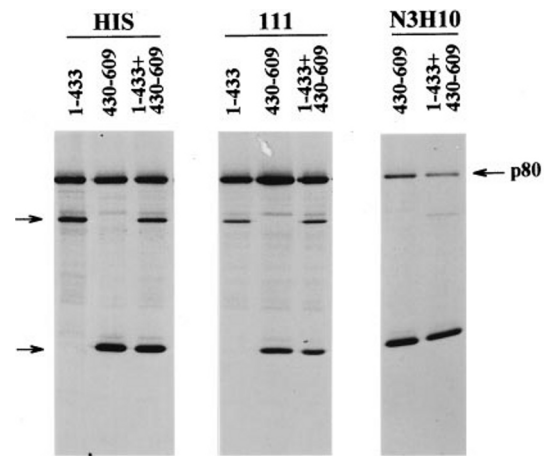*Two Domains of p70 Bind Independently to the p80 Dimer-*
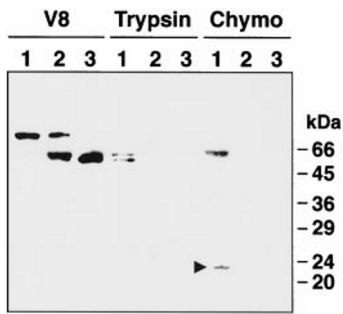
FIG. 3. **Protection of p80 from limited protease treatment by mAb 162.** Ku antigen from K562 cells was affinity-purified onto mAb 162-coated protein A-Sepharose beads, and beads were treated with V8 protease, trypsin, or chymotrypsin (*lane 1*, 2 μg/ml; *lane 2*, 20 μg/ml; *lane 3*, 200 μg/ml). Afterward, the beads were washed and bound fragments analyzed by SDS-PAGE and immunoblotting. The nitrocellulose membrane was probed with mAb S10B1 (anti-p80, amino acids 179–371). Protein fragments recognized by S10B1 are indicated by *arrowhead*. Positions of molecular size standards are shown on the *right*.

FIG. 4. **Co-expression p80 with p70 interaction domains.** Sf9 cells co-infected with p80-bv and recombinant baculoviruses expressing the two interaction domains of p70, either separately or together, were radiolabeled, and cell lysates immunoprecipitated with anti-polyhistidine mAb (*HIS*), anti-p80 mAb (*111*), or anti-p70 mAb (*N3H10*), followed by SDS-PAGE and autoradiography. Positions of p80 and the p70 deletion mutants are indicated.
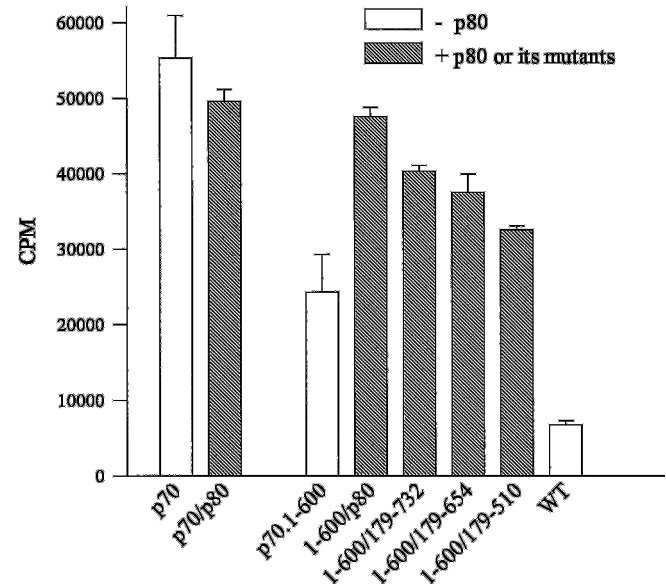
FIG. 5. **DNA immunoprecipitation assay.** Extract from Sf9 cells infected with wild-type baculovirus (*WT*) or recombinant baculoviruses expressing full-length or mutant p70 were immunoprecipitated with mAb N3H10. Binding of the affinity-purified proteins to a 564-base pair ^{32}P-labeled bacteriophage λ *Hin*dIII fragment was tested. Binding of probe to full-length p70 was tested along with the binding of deletion mutant (p70.1–600) (*open bars*). Binding also was evaluated after dimerizing full-length p80 or deletion mutants 179–732, 179–654, or 179–510 to p70.1–600 (*hatched bars*). *WT*, DNA binding activity of cell lysates from Sf9 cells infected with wild-type baculovirus.

ization Site—The p70 protein carries two separate domains (amino acids 1–115 and 430–482) (16) that apparently interact with a single domain of p80 (amino acids 371–510). Heterodimer assembly was evaluated further by co-expressing p70 deletion mutants overlapping the two dimerization domains separately or together with p80, and immunoprecipitating with anti-Ku mAbs (Fig. 4). Protein expression was verified by anti-polyhistidine immunoprecipitation (Fig. 4, *HIS*). When co-expressed individually with p80, p70 (1–433) as well as p70 (430–609) could be co-immunoprecipitated by anti-p80 mAb 111 (Fig. 4, *111*). In addition, both 1–433 and 430–609 were co-immunoprecipitated by mAb 111 when co-expressed together with p80. The bands intensities were similar regardless of whether the p70 proteins were co-expressed with p80 separately (1–433, 430–609) or together (1–433 + 430–609), suggesting that the p70 mutants do not interfere with each other's binding to p80. Anti-p70 mAb N3H10 also co-immunoprecipitated p80 in 430–609/p80 co-expressing lysates. More importantly, N3H10 co-immunoprecipitated 1–433 along with p80. Some free 430–609 also was immunoprecipitated, accounting for the greater intensity of that band (Fig. 4, *right panel*). N3H10 does not immunoprecipitate 1–433 when the mutant protein is expressed alone or co-expressed with 430–609 (data not shown), indicating that the two p70 dimerization domains can bind independently to the same p80 molecule. Similar results were obtained when smaller fragments of p70 and p80 carrying dimerization sites were investigated in the same manner (data not shown). Taken together, these data indicate that the two dimerization domains of p70 interact with the central region of p80 and do not compete with one another for binding.

DNA Interaction Domain of p80—The p70 protein has two DNA binding domains, each partially overlapping one of the dimerization sites. The C-terminal site (amino acids 536–609) has DNA end binding activity by itself, whereas N-terminal DNA binding activity requires dimerization with p80. Partial activity is exhibited by amino acids 1–115, and complete activity by amino acids 1–542. Thus, p70 deletion mutants lacking the p80 independent C-terminal domain, such as 1–600, 1–542, or 1–115, bind DNA in a p80-dependent manner. To determine how the p80-dependent site is assembled, the DNA binding activity of p70 (1–600) co-expressed with a panel of p80 mutants overlapping the dimerization domain of p80 was tested (Fig. 5). It was necessary to use DNA immunoprecipitation assays because binding in gel shift assays requires both DNA binding sites (16). Consistent with previous findings, wild-type p70 by itself bound to linear dsDNA as well as did the p70/p80

heterodimer (Fig. 5, *left*). The p70 deletion mutant 1–600 bound DNA poorly, but clearly better than extract from cells infected with wild-type (*WT*) baculovirus. However, its activity was enhanced considerably by dimerization with p80. Partial p80-dependent DNA binding activity was conferred when p70 (1–600) dimerized with the p80 deletion mutants 179–732, 179–654, or 179–510, each of which overlaps the interaction domain of p80. Decreased binding of the mutants may relate to differences in dimerization efficiency. However, comparable amounts of Ku dimers, determined in preliminary co-immunoprecipitation experiments using N3H10, were used in each lane, arguing that the dimers containing truncated p80 constructs may bind DNA less well. We conclude that p80 (179–
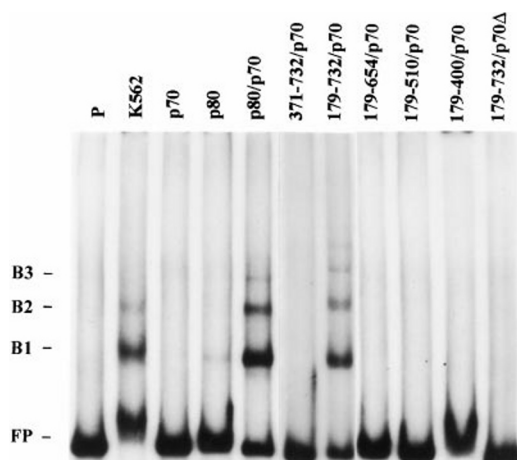
FIG. 6. **DNA binding by gel mobility shift assay.** The same radiolabeled linear DNA probe used for the DNA immunoprecipitation assay (Fig. 6) was incubated with extracts from Sf9 cells infected or co-infected with full-length p70 or p70.1–600 mutant (amino acids 1–600, p70Δ) and full-length or mutant p80. Extracts from human K562 cells also was tested as well as free probe without any cell extract (*lane P*). Binding mixtures were resolved by electrophoresis on a 4% polyacrylamide gel, followed by autoradiography. The positions of free probe (*FP*) and complexes formed with extracts from K562 cells and infected Sf9 cells (*B1–B3*) are indicated.

510) mediates the p80-dependent DNA binding activity of p70.

Role of p80 in Gel Mobility Shift Assay—End binding activity of Ku in gel shift assays requires both the N- and C-terminal DNA binding domains of p70 (16). Because p80 is needed for activity of the N-terminal domain, it also is required for binding in gel shift assays (11, 12). To establish the region of p80 conferring DNA binding activity in gel shift assays, the DNA binding of p80 deletion mutants co-expressed with wild-type p70 was tested. Consistent with previous findings, recombinant Ku heterodimer bound DNA efficiently, whereas p70, p80, and p80 deletion mutants by themselves failed to bind in the gel shift assay (Fig. 6 and data not shown). The p80 mutant 179–732, but not 371–732, 179–654, 179–510, or 179–400, bound DNA after dimerizing with wild-type p70 (Fig. 6). The multiple bands visible on the gel (Fig. 6, *179–732/p70 lane*, *B1*, *B2*, *B3*) suggest that dimerization also restores the internal translocation property of Ku (21). Binding of the recombinant protein 179–732/p70 to DNA was verified by supershift assays using anti-Ku mAb 162 and anti-polyhistidine mAb HIS-1 (data not shown). Paradoxically, we have shown previously that dimerization with wild-type p80 compensates partially for the deletion of a portion (amino acids 601–609) of the p70 C-terminal DNA binding domain (p70 (1–600), p70Δ), even though the intact C-terminal domain binds DNA independently of p80 (16). However, p80 (179–732) failed to restore the DNA binding activity of p70Δ (Fig. 6, *179–732/p70Δ lane*). Thus, p80 (179–732) was required for Ku DNA end binding activity in the gel shift assay, but this region was insufficient to restore the DNA binding activity of p70Δ. Only full-length p80 exhibited the latter effect.

Role of p80 in DNA End Binding—The C-terminal DNA binding domain of p70 recognizes dsDNA termini preferentially (17), and could, by itself, explain the end binding activity of Ku antigen. The role of the p80-dependent DNA binding site has not been defined. To evaluate whether this site also exhibits preferential end binding activity, competition experiments were carried out as detailed in Ref. 17. Consistent with previous findings, binding of the intact p70/p80 heterodimer to a linear double-stranded DNA probe was preferentially inhibited by linear dsDNA (Fig. 7). There was little inhibition of binding by closed circular DNA (φX174 Rf I DNA or pBKS plasmid

DNA) or by tRNA. The full-length p70 protein by itself and p70 (amino acids 430–609) displayed a similar inhibition pattern, suggesting that p70 also binds preferentially to DNA termini. Dimerization of each of the p70 DNA binding domains (p70.430–609 and p70.1–542, respectively) with full-length p80 resulted in constructs that also bound preferentially to linear dsDNA. Binding was not inhibited significantly by φX174 Rf I DNA, pBKS plasmid DNA, or tRNA.

DISCUSSION

Deficiency of Ku autoantigen leads to defective DSBR and V(D)J recombination, resulting in x-ray sensitivity and severe combined immunodeficiency (22). Despite the fact that the p70 Ku subunit can bind dsDNA ends independently of p80 (17), DSBR is absent in cells with p80 mutations (13, 14), apparently due to the existence of a second, dimerization-dependent, DNA binding site (16). In the present study, amino acids 371–510 of p80 was shown to mediate dimerization with p70 and recognition by the Ku dimer-specific mAb 162, in addition to participating in DNA binding. As illustrated in Fig. 8, the locations of the DNA binding and dimerization domains of p80 are consistent with the DSBR-deficient phenotypes of the XR-V9B and XR-V15B cell lines (13). These data also are consistent with the recent finding that heterodimer formation is essential for DSBR *in vivo* (15).

Assembly of Ku Heterodimer—Amino acids 371–510 of p80 were shown to mediate dimerization with p70 (Fig. 1). A "core" domain (amino acids 400–510), along with sequences flanking this region, may play a crucial role in dimerization, because p80 mutants 179–510, 371–510, and 400–732, but not 400–510, can interact with p70. The leucine zipper-like motif near the N terminus of p80 (amino acids 146–178; Fig. 8) (18) is outside of the dimerization domain, consistent with previous observations that it is dispensable for interactions with p70 in the yeast two-hybrid system (12, 23). Similarly, the leucine zipper-like motif of p70 does not mediate dimerization with p80 (16). Although the catalytic subunit of DNA-dependent protein kinase contains an extensive leucine zipper-like motif (24), it is outside the domain that interacts with Ku (25). The leucine zipper-like sequences may interact with other proteins or may not be of functional significance.

The location of the p80 dimerization site reported here is consistent with recent observations of others (23), but less so with other reports (12, 26). Although the region identified here contains the 28-amino acid "minimal functional interaction domain" reported previously (26), the present data indicate that additional sequences are important, either for protein-protein interactions or for promoting folding of the dimerization site. The interaction sequence defined here apparently is distinct from the C-terminal 32-kDa region reported to be important for subunit-subunit interaction in the yeast two-hybrid system (12). The explanation for this discrepancy is unclear.

Three lines of evidence support the idea that the central, rather than C-terminal, portion of p80 interacts with p70. 1) This region is co-immunoprecipitated along with wild-type p70 (Fig. 1); 2) the same region is required for recognition by mAb 162 (Fig. 2), which is specific for the p70/p80 dimer and stabilizes it under dissociating conditions (19); and 3) mAb 162 also protects this region from limited proteolysis (Fig. 3). Together with previous data (16), these data indicate that the conformational epitope recognized by mAb 162 comprises amino acids 430–542 of p70 in association with amino acids 179–510 of p80 (Fig. 8).

An unexpected finding was that the two p70 dimerization domains, amino acids 430–542 and 1–433, respectively, associate with the same region of p80. Moreover, both domains can associate with the same p80 molecule (Fig. 4). The two p70

FIG. 7. **Binding preferences of the two Ku DNA-binding sites.** Extracts from Sf9 cells infected or co-infected with recombinant baculoviruses expressing full-length or mutant p70 or p80 were immunoprecipitated with mAbs N3H10 or 111. Binding of the affinity-purified proteins to a 564-base pair, ^{32}P-labeled bacteriophage λ *Hin*dIII fragment was tested in the presence of 0, 50, or 500 ng of cold competitor (sonicated salmon sperm DNA (linear dsDNA), φX174 (RFI) DNA (closed circular dsDNA), pBKS plasmid DNA (closed circular dsDNA), or yeast tRNA, respectively). Radioactivity remaining on the beads was quantitated by scintillation counting and plotted as percentage of DNA bound without competitor.
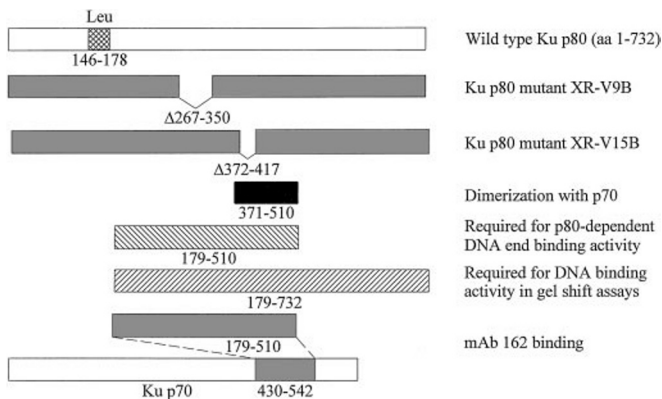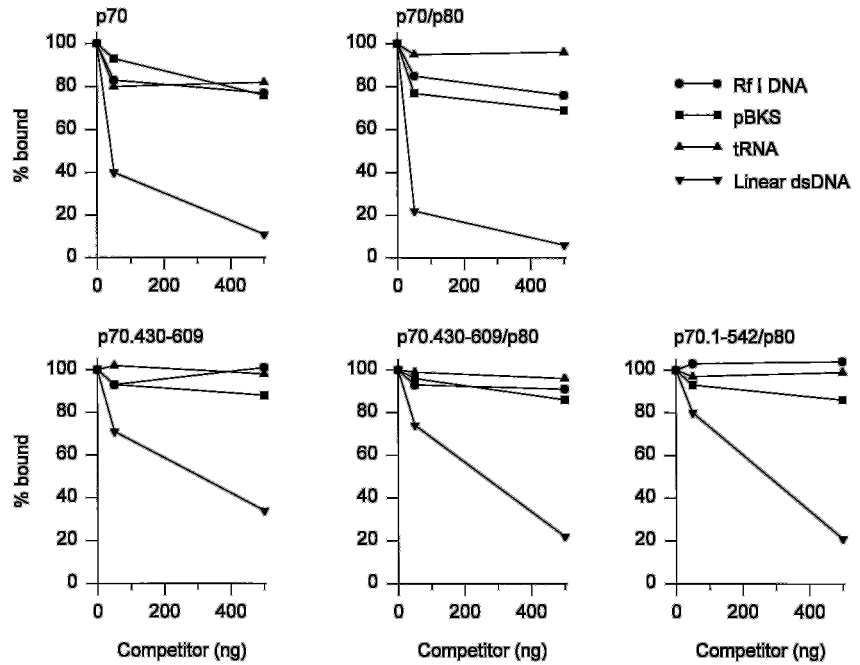
FIG. 8. **Functional domains of p80.** At the *top* is a diagram of the full-length wild-type p80 protein showing the location of a leucine zipper-like sequence (amino acids 146–178) previously identified. Internal deletions reported previously (13) in the radiation-sensitive hamster mutant cell lines XR-V9B (Δ267–350) and XR-V15B (Δ372–417) are diagrammed. The dimerization site identified in Fig. 1 (amino acids 371–510) is indicated by *solid shading*, and regions involved in DNA binding activity as indicated by immunoprecipitation (amino acids 179–510) or gel shift (amino acids 179–732) assays are *diagonally cross-hatched.* A region involved along with p70 (amino acids 430–542) in formation of the 162 epitope (p80 amino acids 179–510) is shown at the *bottom.*

domains may associate with separate sites in the central interaction domain of p80 because the two p70 interaction domains did not appear to compete with one another for binding (see Fig. 4, N3H10 immunoprecipitates). Although the precise location of the two p80 subdomains remains to be determined, the conformational integrity of the core domain (amino acids 400–510) is crucial for binding to p70 because additional amino acids facilitate dimerization and the core domain did not dimerize with p70 by itself (Fig. 1). Recent data indicating that mutations at positions 453 and 454 abrogate the interaction of p80 with p70 (26) are consistent with these observations.

p80-dependent DNA Binding Site of Ku—The interactions of Ku with DNA are complex. End binding activity of Ku is of critical importance for DSBR and V(D)J recombination (15, 22). In addition, internal, sequence-specific binding to a negative regulatory element in the long terminal repeat of mouse mammary tumor virus has been reported (8) as well as interactions

with single-stranded DNA or other substrates (21). Thus, like many other DNA binding factors, Ku antigen may be multifunctional (27).

At least two different sites interact with DNA (Fig. 9). A p80-independent site is located on the C-terminal end of p70 and binds preferentially to dsDNA ends (16, 17). The N-terminal portion of p70 forms a second site upon dimerization with p80. Neither site by itself is sufficient for DNA binding activity in gel mobility shift assays, but together they confer activity (16). A region overlapping the minimal dimerization domain of p80 confers p80-dependent DNA binding activity (Fig. 5). Interestingly, a much larger portion of p80, including the intact C terminus (amino acids 179–732), probably is needed for DNA binding in gel mobility shift assays (Fig. 6). This site, like the p80-independent site, binds preferentially to dsDNA ends (Fig. 7).

Models for the Interaction of Ku with DNA—The existence of two DNA end binding sites may have implications for the function of Ku. Although it is clear that Ku is an integral factor in DSBR, its precise role remains uncertain. Two models have been proposed: 1) that Ku protects DNA ends from exonucleolytic degradation, and 2) that it plays an active role in the rejoining process. The first possibility is suggested by the fact that imprecise joining occurs at higher frequency in cells with mutant Ku (28, 29). However, the direct role of Ku antigen in non-homologous, and not homologous, recombination (30) strongly supports the second model. In addition, it recently has been shown that Ku increases the frequency of accurate end joining mainly by increasing the ligation rate, not by protecting ends from degradation (31). Moreover, p80 is not required for the protection of DNA ends during V(D)J recombination in Ku80 knockout mice (32). Thus, the available evidence favors the idea that Ku antigen physically juxtaposes broken DNA ends, enhancing the activity of eukaryotic DNA ligases (31). This model also is consistent with atomic force microscopy data indicating that Ku can join together two DNA ends (33–35). It has been proposed that, upon interacting with two DNA termini, tetramerization of Ku may facilitate their juxtaposition (33). Our data suggest an alternative model: that a single Ku heterodimer might link two DNA ends, helping to ensure that they are aligned properly for ligation (Fig. 9). This model is supported by the atomic force microscopy studies, suggesting
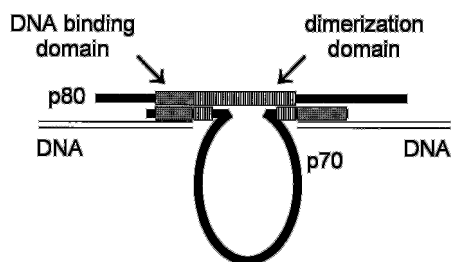
Fig. 9. **Model of the structure of Ku antigen.** A central p80 dimerization domain p80 interacts with two dimerization sites near the C- and N termini of p70, respectively. Interaction of p80 with the N-terminal dimerization site of p70 promotes the formation of a p80-dependent DNA binding site. A second DNA binding site in the C-terminal portion of p70 is p80-independent. Both sites bind preferentially to DNA ends, suggesting that a single molecule of Ku may juxtapose two DNA termini, facilitating their ligation by other components of the DNA repair apparatus.

that the size of the Ku protein is consistent with the presence of a single Ku heterodimer at each DNA junction (35). Nevertheless, further studies are needed to verify that Ku joins DNA fragments as a dimer, instead of a tetramer. We cannot exclude the possibility that two binding sites are needed in order for Ku to properly contact a single DNA end. For instance, it is possible that the first site binds DNA and then slides along it, whereas the second site allows Ku to pause at the DNA end. A similar two-site model may explain the binding of p53 to single-stranded DNA ends and internal segments (36). The two sites also could confer the ability to bind different DNA or RNA substrates (8, 37), although so far we have not obtained evidence for this possibility in competition studies similar to those in Fig. 7.

Molecular Basis of Ku Mutations—The present data may be relevant to the phenotypes of mutant hamster cell lines in the x-ray cross-complementing (XRCC) 5 group. Two XRCC5 group mutant cell lines, XR-V15B and XR-V9B, are deficient in both DSBR and V(D)J recombination (13). They have internal in-frame deletions of 84 and 46 amino acids, respectively (13), resulting in the deletion of amino acids 267–350 (XR-V9B) and 372–417 (XR-V15B) of p80 (Fig. 8). In view of the locations of the p70 interaction domain (amino acids 371–510) and a region involved in p80-dependent DNA binding (amino acids 179–510), XR-V9B may be deficient in DSBR because of an inability to form the p80-dependent DNA binding site. Dimerization should occur normally. In contrast, our data predict that p80 mutant XR-V15B may be inactive in DSBR because it cannot dimerize with p70 (Fig. 8).

The p80 mutants described by Singleton *et al.* (14) also are of interest with respect to the dimerization and DNA binding domains reported here. Deletion mutants lacking the N-terminal 40, 69, or 114 amino acids of p80 had undetectable DNA end binding activity and were radiosensitive. However, the present data suggest that p70 dimerization and DNA binding activity (both by immunoprecipitation and gel shift assays) of these mutants should be intact. Interestingly, although the three mutants had wild-type or reduced levels of p80 protein, all had low levels of p70 compared with wild-type cells (14). Dimerization with p70 prevents the rapid degradation of p80 in K562 cells (38). In other cell lines, dimerization stabilizes both proteins (1, 13). The low levels of p70 in N-terminally truncated p80 mutants (14) raise the possibility that sequences located near the N terminus may play a role in the mutual stabilization of p70 and p80 in certain cell lines, even though this region is dispensable for dimerization. Thus, it is conceivable that the radiosensitivity of the mutants described by Singleton *et al.* reflects enhanced degradation of Ku rather than lack of function (dimerization or DNA binding). Additional studies are

needed to examine whether sequences located outside the minimal dimerization domain play a role in protecting the heterodimer from degradation.

In summary, we have defined regions of p80 mediating dimerization with p70 as well as p80-dependent DNA binding activity. The functional domains identified here may help to explain the phenotypes of radiosensitive XRCC5 mutants. More importantly, the two DNA binding domains both bind dsDNA ends preferentially, consistent with the idea that Ku antigen acts by physically apposing two broken DNA ends, facilitating their ligation during DSBR (30, 31, 33, 35).

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