Role of a Major Autoepitope in Forming the DNA Binding Site of the p70 (Ku) Antigen

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Summary

The Ku antigen is a heterodimer consisting of 70- and 80-kD protein subunits that binds to termini of double-stranded DNA. DNA binding appears to be mediated partly by the 70-kD (p70) subunit, but the precise mechanism of its association with DNA is unclear. High-titer autoantibodies in sera from certain patients with systemic lupus erythematosus recognize at least eight distinct epitopes of Ku, and inhibit DNA binding. In the present studies, the binding of DNA to truncated p70 fusion proteins was determined in Southwestern blots and DNA immunoprecipitation assays. Appropriate folding of the p70 protein was crucial for efficient DNA binding. The minimal DNA binding site, amino acids 536–609, contains a major conformational autoepitope of p70 (amino acids 560–609). Deletion of amino acids 601–609, or substitution of ala-ala-ala for lys-ser-gly at positions 591–593, eliminated DNA binding as well as autoantibody binding, suggesting that the same secondary or supersecondary structure is involved in both DNA binding and autoantibody recognition. Residues within the DNA binding site/autoepitope closely resemble the helix-turn-helix motif in bacteriophage λ Cro protein and certain other DNA binding proteins, and mutations predicted to destabilize this structure eliminated DNA binding. Adjacent to the helix-turn-helix is a highly basic domain (positions 539–559) that was also required for DNA binding. The findings suggest that the DNA binding site of p70 consists of a basic domain adjacent to a helix-turn-helix structure that also forms a major autoepitope.

Sera from certain patients with SLE and overlap syndromes contain high titers of autoantibodies to the Ku antigen, a dimer consisting of 70- and ~80-kD protein subunits (p70 and p80, respectively) that binds to termini of double-stranded DNA (1–4). The function of Ku is not known, but a role in DNA replication/repair (4, 5) or transcriptional activation (6–8) has been proposed.

We previously defined two autoepitopes of the human p70 protein, located on amino acids (aa)[1] 560–609 (epitope 1), and 506–535 (epitope 2), respectively (9, 10). Epitope 1 is recognized by autoantibodies more frequently than epitope 2. Since most autoepitopes recognized by lupus autoantibodies are highly conserved between species, it has been proposed that autoantibodies may bind preferentially to certain active or functional sites (11), such as DNA binding sites. The competitive inhibition of DNA binding by anti-Ku antibodies (4, 12) is consistent with that possibility. However, unlike most autoepitopes, epitope 1 is poorly conserved between humans and mice (10). In the present studies, we demonstrate that p70 contains a ~75 amino acid DNA binding domain (residues 536–609) composed of epitope 1 and an adjacent basic domain. In addition, epitope 2 may contribute to the efficiency of DNA binding. Correct folding of this region is necessary for both DNA binding and autoantibody recognition.

[1] *Abbreviation used in this paper:* aa, amino acids.

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Materials and Methods

Antibodies. Patient JM serum is specific for the epitope 1, and has little reactivity with epitope 2. Murine mAbs 162 and N3H10 were reported previously (3, 7). 162 is specific for the p70/p80 heterodimer (our unpublished data), and N3H10 is specific for p70; both mAbs react selectively with human Ku antigens. 162 is of the IgG2a subclass (3), and N3H10 is an IgG2b (W. H. Reeves and S. Craven, unpublished data). mAbs were purified by ammonium sulfate precipitation from ascitic fluid as described previously (3). A murine IgG1 anti-TrpE mAb was obtained from PharMingen (San Diego, CA).

Fusion Proteins. Amplification of specific fragments of p70

cDNAs by the PCR, insertion of these fragments into pATH vectors, and the expression of TrpE-p70 and TrpE-p80 fusion proteins in *Escherichia coli* were described previously (9). The following TrpE-p70 fusion proteins were tested: 77 (p70 amino acids 419–609), N (aa 469–600), P (aa 506–600), R (aa 506–535), E (aa 536–609), and A (aa 560–609). Fusion proteins N′ (p70 aa 469–609) and P′ (aa 506–609) were generated by the same procedures. A full-length TrpE-p80 fusion protein (20a) (9, 13) was also tested.

Production of fusion proteins by *E. coli* RR1 cells harboring plasmids of interest was induced by indoleacrylic acid as described previously (9). Fusion proteins were solubilized by incubating the bacteria for 10 min in ice cold 50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0, containing 10 mg/ml lysozyme, followed by the addition of NP-40 to 0.5%, NaCl to 0.5 M, PMSF to 0.5 mM, and Trasylol (0.3 trypsin inhibitor U/ml). The extracts were incubated for 30 min at 0°C, sonicated briefly to shear DNA, and centrifuged at 10,000 *g* for 10 min. The soluble and insoluble fractions were analyzed by SDS-PAGE and immunoblotting for fusion protein content, and soluble fusion proteins were adjusted to approximately the same concentration.

Mutagenesis. Site-directed mutagenesis for generating the p70 P′ (A) mutant fusion protein was performed essentially according to the method of Landt et al. (14). The sequences of the left and right flanking primers were 5′-GCGAATTCCTGACATTGCCCAAGG-3′ and 5′-GTAAAACGACGGCCAGT-3′, respectively. The sequence of mutagenic primer was 5′-GCTTACGGGCTGGCGGCTGCTCTGAAGAAGCAG-3′. The first PCR was carried out using the mutagenic and right flanking primers. The resulting DNA fragment was separated on a 1% low-melting agarose gel, and the gel slice containing this fragment was used in the second PCR without further purification. The primers used in the second PCR were the left flanking primer and the entire intermediate fragment from the first PCR. The fragments from the second PCR were digested with EcoRI and subcloned into the pATH 11 vector. Expression of the mutant fusion protein followed the same procedures described above.

Immunoblotting. Immunoblot analysis of the fusion proteins was performed using human autoantibodies or murine mAbs as described (9, 10). Human autoimmune serum was diluted 1:500, and ascitic fluid from the N3H10 and anti-TrpE mAbs were diluted 1:1,000 for probing immunoblots. Second antibodies were alkaline phosphatase–conjugated goat anti–human IgG and goat anti–mouse IgG antibodies for detecting human autoantibodies and murine mAbs, respectively.

Southwestern Blot. The Southwestern blot procedure described by Allaway et al. (15) was used with minor modifications. Briefly, fusion proteins were fractionated on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes using standard procedures (9). The blots were renatured for 18–24 h with three changes of 5% nonfat dry milk, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 2.5% NP-40, 1 mM DTT, and 0.1 mM $ZnCl_2$. In some experiments, the effect on DNA binding of omitting 0.1 mM Zn^{2+} from the renaturation buffer was tested. After renaturation, the blots were probed in 0.25% nonfat milk, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT for 3 h with either: (*a*) ^{32}P end-labeled bacteriophage λ HindIII fragments (0.5, 2.0, or 2.2 kb; 10^6 cpm/ml); or (*b*) 2.0-kb HindIII fragment of bacteriophage λ labeled with [^{32}P]dCTP by random priming (16). Blots were washed three times each 30 min with 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, and exposed to x-ray film.

Immunoprecipitation Assay for DNA Binding. This assay was performed as described previously (3, 12, 17, 18), with minor modifications. Protein A–Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) (20 μl of a 50% slurry in water) were coated with the mAbs (5 μl of ~1 mg/ml mAb in PBS [150 mM NaCl, 20 mM sodium phosphate, pH 7.5]) for 3 h at 22°C. IgG2a and IgG2b mAbs (162 and N3H10, respectively) were bound directly to the beads; the IgG1 anti-TrpE mAb was attached to the beads using rabbit anti–mouse Ig antibodies (10 μl of 1.0 mg/ml DEAE-cellulose purified antibody in PBS). The antibody-coated beads were washed three times with 0.15 M NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA, 0.5% NP-40, 1 mg/ml OVA, 0.02% NaN_3 before incubating for 3 h with bacterial lysates containing fusion protein (see above). The beads were then washed twice with mixed micelle buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA, 0.25 M sucrose, 0.5% SDS, 2.5% Triton X-100), followed by two washes in 0.5 M NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA to elute cellular DNA (12), and two washes in 0.15 M NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA.

p70 and p80 fusion proteins were tested for binding to DNA by incubating the immunoaffinity-purified Ku antigens on protein A–Sepharose beads with ^{32}P end-labeled DNA fragments. A HindIII digest of bacteriophage λ DNA was end labeled with [^{32}P]dATP (~2,000 Ci/mmol; New England Nuclear, Boston, MA) using Klenow fragment (19). 100 ng of labeled DNA was incubated for 1 h with the antigen-coated beads in binding buffer (0.15 M NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA, 50 μg/ml methylated BSA). The beads were then washed twice with mixed micelle buffer, and three times with 0.15 M NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA, then resuspended in 50 μl of the same buffer containing 100 μg/ml proteinase K (International Biotechnologies Inc., New Haven, CT). The beads were incubated for 2 h at 37°C, and then extracted with phenol. Carrier RNA (1 mg tRNA) was added, and the nucleic acids were ethanol precipitated and analyzed by electrophoresis on 1.0% agarose gels. The gels were fixed for 20 min in 10% TCA, dried, and autoradiographed.

Results

Previous studies have demonstrated that the intact Ku (p70/p80) complex binds DNA in vitro (3), and Southwestern blots and UV crosslinking experiments suggest that this binding may be mediated, at least in part, by the p70 subunit (4, 7, 15, 20). Our initial attempts to confirm selective binding of ^{32}P-labeled bacteriophage λ DNA fragments to the cellular p70 protein or p70 fusion proteins by Southwestern blotting were unsuccessful. However, the conditions reported by Allaway et al. (15), which include an extended period of renaturation, permitted efficient binding of end-labeled DNA fragments to both the 77 fusion protein (p70 amino acids 419–609 fused to the TrpE protein) (Fig. 1 *A*) and to the cellular p70 protein (not shown). No binding to the TrpE protein alone was detected. Although Zn^{2+} was present in the original renaturation buffer (15), it was not required for DNA binding (Fig. 1 *A*, compare *top* and *bottom*). The ~2.0- and 2.2-kb HindIII fragments of bacteriophage λ bound to the immobilized 77 fusion protein somewhat more efficiently than the ~0.5-kb fragment, despite having identical (HindIII site) recessed termini. Patient JM serum bound efficiently to the 77 fusion protein on immunoblots, but not to the TrpE fusion protein (Fig. 1 *B*), whereas the anti-TrpE mAb displayed equivalent binding to both the 77 fusion protein and to the TrpE protein, indicating that approximately equal amounts of 77 and TrpE fusion protein were transferred to nitrocellulose.

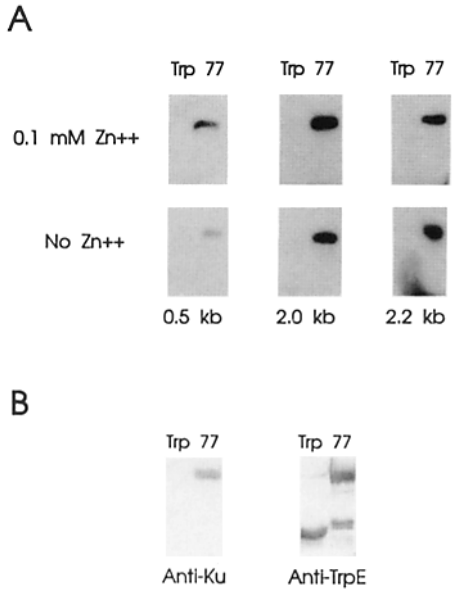

Figure 1. Binding of DNA to p70 fusion protein. (*A*) Southwestern blot. Fusion protein production by *E. coli* RR1 cells harboring plasmids pATH 11 and 70.77-pATH 11 was induced by indoleacrylic acid, and bacterial lysates containing TrpE (Trp) or 77 fusion protein were separated by SDS-PAGE and transferred to nitrocellulose. Blots were incubated in renaturation buffer in the presence or absence of 0.1 mM $ZnCl_2$, and probed with end-labeled bacteriophage λ DNA fragments (10^6 cpm/ml). Bacteriophage λ probes: 0.5-kb HindIII fragment; 2.0-kb HindIII fragment; 2.2-kb HindIII fragment. Binding of the 2.0- and 2.2-kb fragments was more efficient than that of the 0.5-kb fragment in the presence of equal amounts (cpm) of labeled probe. The presence or absence of Zn^{2+} in the renaturation buffer made little difference in binding efficiency. (*B*) Immunoblots with patient JM serum and anti-TrpE mAb. Nitrocellulose blots from *A* were probed with anti-Ku autoimmune serum (patient JM, 1:500 dilution) or anti-TrpE mAb (1:1,000 dilution). Binding of anti-TrpE antibodies was detected by 1:1,500 alkaline phosphatase–conjugated goat anti-mouse IgG antibodies; binding of human autoantibodies was detected by 1:1,500 alkaline phosphatase–conjugated goat anti-human IgG antibodies. JM serum bound to the 77 fusion protein, but not to TrpE. Staining with the anti-TrpE mAb demonstrated that equivalent amounts of the 77 and TrpE fusion proteins were transferred to nitrocellulose.

To more precisely localize the DNA binding site, the binding of DNA labeled with [^{32}P]dCTP by random priming (16) to a panel of p70 fusion proteins was examined by Southwestern blotting (Fig. 2 *A*). In addition to fusion protein 77, fusion proteins P′ (aa 506–609) and E (aa 535–609) bound DNA, whereas fusion proteins P (aa 506–600), A (aa 560–609), and R (aa 506–535) did not (Fig. 2 *A*). No binding of DNA to the TrpE protein (Fig. 2 *A*, *Trp*) was observed. Binding to fusion proteins 77 and P′ was somewhat stronger than binding to fusion protein E. Binding to a full-length p80 fusion protein (Fig. 2 *A*, *20a*) was not detected, consistent with previous observations that p80 does not bind DNA on Southwestern blots (4). Immunoblot analysis using the anti-TrpE mAb indicated that the amounts of fusion proteins in different lanes was similar (Fig. 2 *B*). Comparison of the Southwestern blot (Fig. 2 *A*) and immunoblot (Fig. 2 *B*) patterns suggested that although proteolytic degradation of some fusion proteins occurred, only the full-length 77, P′, and E proteins (and not their proteolytic degradation fragments) bound DNA. This is most likely related to the observation that short deletions from the COOH terminus of p70 (compare fusion proteins P′ and P, Fig. 2 *A*) eliminate DNA binding. Thus, aa 536–609 are critical for DNA binding on Southwestern blots, whereas the inclusion of aa 506–535 may enhance binding somewhat (Fig. 3).

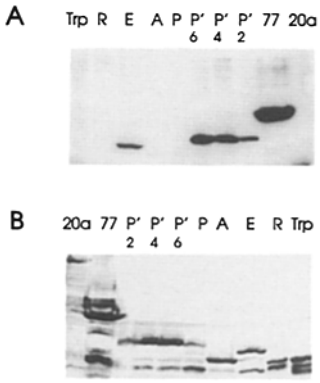

Figure 2. Binding of DNA to p70 fusion proteins. (*A*) Southwestern blot. Approximately equal amounts of TrpE-p70 fusion proteins R (aa 506–535), E (aa 536–609), A (aa 560–609), P (aa 506–600), P′ (aa 506–609), and 77 (aa 419–609) were tested for DNA binding after renaturation. The binding of different amounts of fusion protein P′ ranging from 2 to 6 μl was examined. The TrpE protein (Trp) and a full-length p80-TrpE fusion protein (20a) were also tested. The blot was probed with 50 ng of 2.0-kb bacteriophage λ HindIII fragment labeled with [^{32}P]dCTP by the random primer method. (*B*) Immunoblot with anti-TrpE mAb. Identical blot to that shown in *A* was probed with anti-TrpE mAb (1 μg/ml), followed by 1:1,500 alkaline phosphatase–conjugated goat anti-mouse IgG antibodies to demonstrate that approximately equal amounts of fusion proteins were transferred. Proteolytic degradation fragments of fusion proteins, which react with anti-TrpE mAb, do not bind DNA in *A*.

Taken together, the Southwestern blots suggest that renaturation of p70 is required for efficient DNA binding, and that the lack of a prolonged renaturation step was responsible for our initial failure to detect DNA binding to p70 on Southwestern blots. These experiments permitted the DNA binding site of p70 to be localized to a region containing the major autoepitope (epitope 1) of p70 (Fig. 3), which, like the DNA binding site, is highly dependent on protein folding (9, 10).

In view of the importance of protein folding in the p70-DNA interaction, we also examined DNA binding to non-denatured Ku proteins in an immunoprecipitation assay used previously in studies of the Ku-DNA interaction (3, 12) and

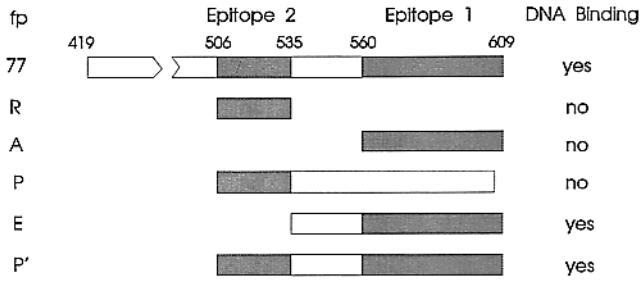

Figure 3. Diagram of p70 fusion proteins and their DNA binding properties. Fusion proteins 77 (aa 419–609), R (aa 505–535), A (aa 560–609), P (aa 506–600), E (aa 536–609), and P′ (aa 506–609) are illustrated. Positions of epitopes 1 and 2 (aa 560–609 and 506–535, respectively) are indicated. Integrity of epitopes 1 and/or 2 (defined by reactivity with human autoantibodies) in individual fusion proteins is designated by shading. DNA binding to the fusion proteins by Southwestern blot and DNA immunoprecipitation assay is indicated on the right.

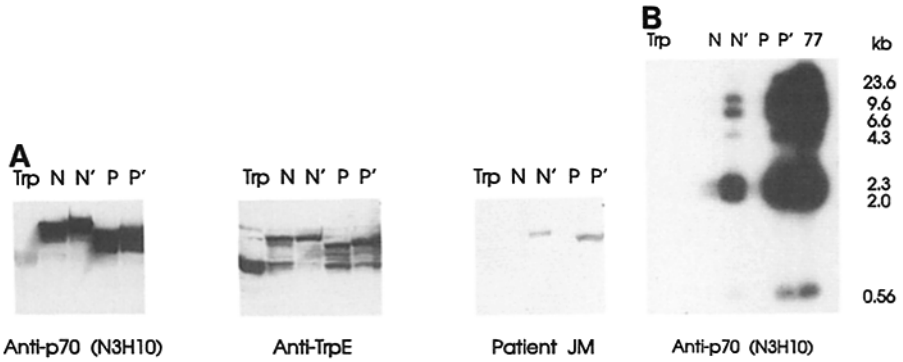

Figure 4. Binding of DNA to nondenatured p70 fusion proteins. (*A*) Immunoblot of solubilized *E. coli* proteins, p70 fusion proteins N (aa 469–600), N′ (aa 469–609), P (aa 506–600), P′ (aa 506–609), and TrpE were solubilized under nondenaturing conditions. Aliquots of each soluble fusion protein preparation were analyzed by SDS-PAGE and immunoblotted. Blots were probed with anti-p70 mAb N3H10 (~1 μg/ml, *left*), anti-TrpE mAb (~1 μg/ml, *middle*), followed by 1:1,500 alkaline phosphatase–conjugated goat anti-mouse IgG antibodies, or with autoimmune serum from patient JM (1:500 dilution, *right*) containing antibodies to epitope 1 but not epitope 2, followed by 1:1,500 alkaline phosphatase–conjugated goat anti-human IgG antibodies. Solubilization of fusion proteins TrpE, N, N′, P, and P′ was comparable as determined by reactivity with anti-p70 (N3H10) and anti-TrpE mAbs. Fusion proteins N′ and P′ reacted with JM serum, whereas fusion proteins N and P did not, indicating that deletion of aa 601–609 eliminates autoantibody binding to epitope 1. (*B*) DNA immunoprecipitation assay. Bacterial lysates (*E. coli* RR1) containing fusion proteins N, N′, P, P′, 77, and TrpE were subjected to affinity purification on mAb N3H10–coated protein A-Sepharose beads. Binding of ^{32}P-end-labeled DNA fragments (bacteriophage λ HindIII digest) was determined by DNA immunoprecipitation assay. Note that fusion proteins N and P did not bind DNA, and that the 2.3- and 2.0-kb HindIII fragments bound to the 77, N′, and P′ beads more efficiently than the 0.56-kb fragment.

for analysis of sequence-specific DNA binding by other factors (17, 18). Recombinant p70 and p80 proteins were isolated under conditions (0.5 M NaCl and 0.5% NP-40) less harsh than those used in the Southwestern blot experiments. Under these nondenaturing conditions, at least partial solubilization of many p70 fusion proteins was observed (Fig. 4 *A*), whereas p80 fusion proteins were solubilized poorly, and could not be analyzed in this assay (data not shown). mAb N3H10 and the anti-TrpE mAb bound to fusion proteins N (aa 469–600), N′ (aa 469–609), P (aa 506–600), and P′ (aa 506–609) (Fig. 4 *A*), as well as 77 (not shown) from extracts solubilized under nondenaturing conditions. In contrast, patient JM serum bound to fusion proteins N′ and P′, but not N or P, in agreement with previous observations that deletion of aa 601–609 destroys antigenicity of p70 epitope 1 (9).

Comparable amounts (Fig. 4 *A*) of fusion proteins N, N′, P, P′, and 77 were affinity purified using N3H10 and tested for DNA binding in the immunoprecipitation assay using a mixture of ^{32}P end-labeled HindIII fragments of bacteriophage lambda (Fig. 4 *B*). In agreement with the Southwestern blot data, fusion proteins 77, N′, and P′ bound DNA efficiently, whereas fusion proteins N and P did not, suggesting that aa 601–609 are required for efficient DNA binding. As also noted in the Southwestern blots, there appeared to be preferential binding of the 2.0- and 2.3-kb HindIII fragments to the fusion proteins.

Since N3H10 does not react with fusion proteins A, E, or R (not shown), comparable amounts (Fig. 5 *B*) of these fusion proteins were affinity purified from bacterial lysates using the anti-TrpE mAb, and tested for DNA binding in immunoprecipitation assay (Fig. 5 *A*). As before, fusion proteins 77 and P′ bound DNA efficiently after affinity purification by anti-TrpE, whereas the binding to fusion protein E was reduced compared with fusion proteins 77 and P′, and fusion proteins A, P, and R did not bind DNA.

The DNA immunoprecipitation studies confirmed that aa 536–609 constitute a minimal fragment of p70 required for DNA binding, and that aa 506–535 may increase the efficiency of binding. aa 419–505 may have an additional effect on binding efficiency (compare binding with fusion proteins 77 and P′). Thus, the minimal DNA binding site is composed of the major autoepitope (epitope 1) of p70 plus an adjacent short, strongly basic sequence (Fig. 3). Epitope 2 may also contribute to binding, but is not essential. Deletion of aa 601–609 eliminates both DNA binding (Fig. 4 *B*) and antigenicity of epitope 1 (9; and Fig. 4 *A*). A search for possible DNA binding motifs within this region revealed a sequence (aa 585–603) resembling the helix-turn-helix motifs of a group of other DNA binding proteins (Fig. 6 *A*). The positions of hydrophobic (h) and polar (p) residues within this region

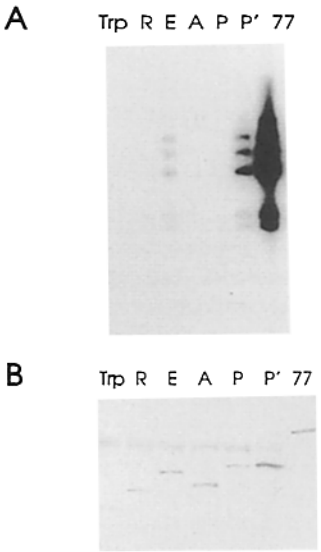

Figure 5. DNA immunoprecipitation analysis of fusion proteins. (*A*) DNA immunoprecipitation assay. TrpE fusion proteins R, E, A, P, P′, and 77, and TrpE alone, were solubilized under nondenaturing conditions, affinity purified with anti-TrpE mAb, and tested for DNA binding in the immunoprecipitation assay. As was the case in Southwestern blot experiments, fusion proteins 77, P′, and E bound DNA, whereas fusion proteins R, A, and P did not. Binding to 77 and P′ was more efficient than to fusion protein E. (*B*) Immunoblot of affinity-purified fusion proteins. Fusion proteins were solubilized under nondenaturing conditions, and affinity-purified in exactly the same manner as in *A*. The affinity-purified proteins were eluted by boiling in SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose. The blot was probed with anti-TrpE mAb (1 μg/ml) followed by 1:1,500 alkaline phosphatase–conjugated goat anti-mouse IgG antibodies.

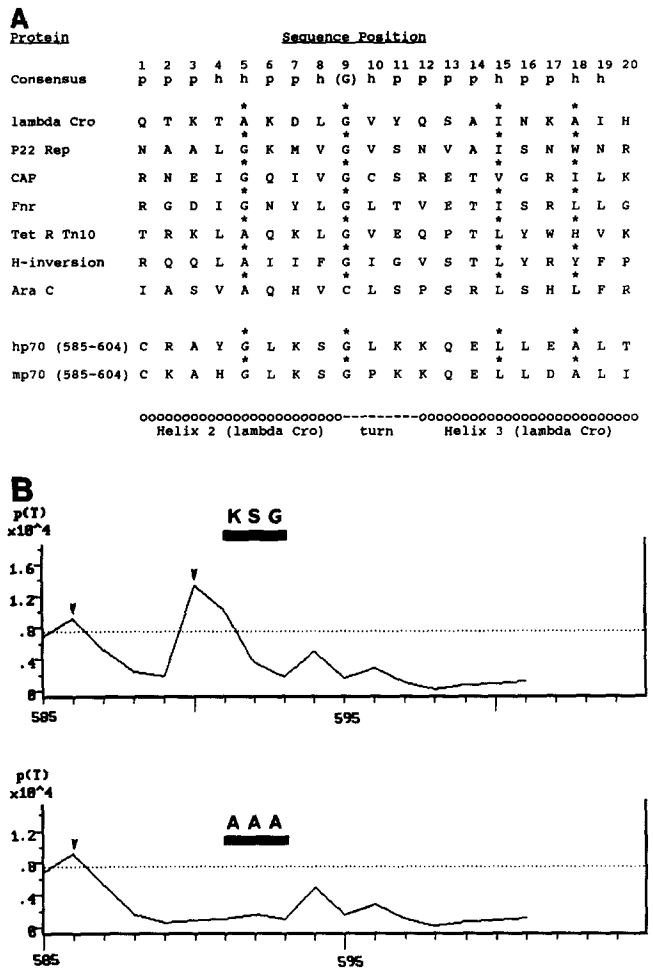

Figure 6. Potential helix-turn-helix motif of p70. (*A*) Alignment of p70 protein sequence with DNA binding proteins containing turn-helix-turn motif. Sequences (and alignments) of known helix-turn-helix proteins were obtained from reference 23, and aligned with the human and murine p70 sequences (hp70 and mp70, respectively) from position 585–604. Positions of helices 2 and 3 of the λ Cro protein are indicated below; h, hydrophobic; p, polar. (*B*) Prediction of p70 secondary structure. β turn probability [*p(t)*] was determined by the method of Chou and Fasman (25). Positions of probable β turns [$p(t) > 0.75 \times 10^4$] are indicated by arrowheads. (*Top*) Secondary structure prediction for p70 (aa 585–609); (*bottom*) secondary structure prediction for p70 (aa 585–609) after altering aa 591–593 from K-S-G (wild-type sequence) to A-A-A. Note that the K-S-G → A-A-A substitution eliminates the predicted β turn near position 590.

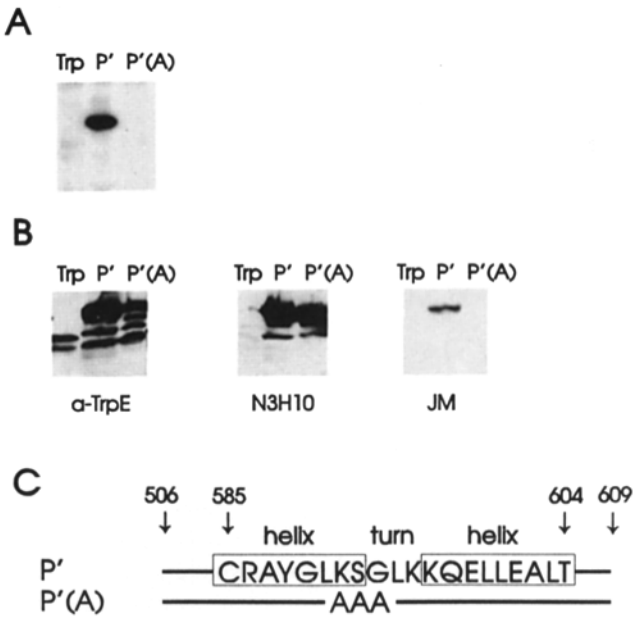

Figure 7. Effect of p70 mutations on DNA binding. (*A*) Southwestern blot. Binding of DNA to similar amounts of TrpE fusion proteins P' (aa 506–609) and P' (A) (aa 506–609 with 591–593, A-A-A) was determined by Southwestern blot. Trp, TrpE protein alone. (*B*) Immunoblots of fusion proteins. Blots similar to those used for Southwestern blot analysis were probed with ~1 μg/ml anti-TrpE mAb (α-TrpE) or anti-p70 mAb (N3H10). Anti-TrpE mAb reacted with all three proteins, TrpE, P', and P' (A), whereas N3H10 reacted only with the TrpE-p70 fusion proteins P' and P' (A). Autoimmune serum from patient JM (1:500 dilution) reacted with fusion protein P', but not with P' (A), indicating that although the N3H10 epitope remained intact in the P' (A) mutant, antigenicity of epitope 1 was destroyed by altering aa 591–593 from K-S-G to A-A-A. (*C*) Diagram of fusion proteins P' and P' (A). Position of possible helix-turn-helix is indicated, as are the positions of the amino acid substitutions introduced in fusion protein P' (A).

were well conserved between the human (21, 22) and murine (10) p70 proteins, the λ Cro protein, and a series of other helix-turn-helix proteins (23, 24), as was the glycine residue at position 9. In addition, Chou-Fasman secondary structure analysis (25) of the p70 sequence was consistent with a β turn at positions 590–593 [p(t) = 1.33] with flanking short α helical stretches, as would be expected in a helix-turn-helix protein (24) (Fig. 6 *B*).

To examine further whether aa 585–603 might form a helix-turn-helix involved in DNA binding, aa 591–593 (lys-ser-gly) of fusion protein P' were converted to ala-ala-ala by site-directed mutagenesis (Fig. 7 *C*, *P' (A)*). Alanine substitutions were chosen in order to minimize effects on the backbone structure of the protein (26). The substitution of three alanines for lys-ser-gly at positions 591–593 was predicted by Chou-Fasman secondary structure analysis (25) to eliminate the β turn near position 590 that was predicted in the wild-type p70 protein (Fig. 6 *B*). Fusion protein P' (A) (p70 aa 506–609, ^{591}ala-^{592}ala-^{593}ala, fused to TrpE) retained reactivity with the anti-TrpE mAb (Fig. 7 *B*, *left*) and mAb N3H10 (*middle*), but was unreactive with autoimmune serum from patient JM (*right*), suggesting that these residues are crucial for antigenicity of the 560–609 epitope. The mutant fusion protein also failed to bind DNA on Southwestern blots, indicating that aa 591–593 are critical for DNA binding as well as autoantibody recognition (Fig. 7 *A*).

Discussion

The Ku autoantigen is a DNA binding protein heterodimer with uncertain function. A role in DNA repair, replication, or transposition has been proposed based on its affinity for termini of double-stranded DNA (4, 27). Other studies have suggested an association with transcriptionally active chromatin (6) and sequence-specific DNA binding activity (7, 8). The mechanism of interaction between Ku and DNA is

incompletely understood. The binding of Ku to DNA is inhibited in vitro by certain human autoimmune sera, suggesting that the DNA binding site is located in close proximity to one or more autoepitopes (4, 12). At least eight autoepitopes of the Ku particle, located on p70, p80, or the p70/p80 heterodimer, were identified previously (9). However, which of these epitopes is located near the DNA binding site was unknown. In the present studies, we show that the DNA binding site of Ku is composed of the major autoepitope of p70 (aa 560–609) plus an adjacent, strongly basic, domain (aa 536–559).

Previously, Southwestern blot analysis of full-length p70 and p80 proteins suggested that the association of Ku with DNA is mediated exclusively by the p70 subunit (4), but more recent studies suggest that both subunits are required for DNA binding in a gel shift assay (28). In addition, both p70 and p80 can be crosslinked to DNA by UV light (7, 8, 20). The present data showing DNA binding to p70, but not p80, fusion proteins (Fig. 2) on Southwestern blots and in DNA immunoprecipitation assays are consistent with an important role of p70 in DNA binding, but do not rule out the possibility that p80 might also contribute to DNA binding, as suggested by Griffith et al. (28). The present results suggest that the DNA binding site of p70 is located on the COOH-terminal 75 aa of p70 (Fig. 3). Since nonspecific binding of DNA to proteins on nitrocellulose membranes is a potential drawback of the Southwestern blot technique, we also examined DNA binding by nondenatured p70 in DNA immunoprecipitation assays (Figs. 4 and 5), with comparable results. Of course, charge-charge interactions might still be responsible for the binding of DNA to p70 fusion proteins in these assays. However, control experiments established that there was little or no binding of DNA to the TrpE portion of the fusion proteins. Moreover, the striking difference in DNA binding between N and N′ and P and P′, respectively, also suggests strongly that binding of DNA to the fusion proteins was not mediated by charge-charge interactions alone. Deletion of aa 601–609 severely perturbs the secondary or supersecondary structure of the COOH terminus of p70, as indicated by a complete loss of reactivity with autoantibodies to epitope 1 (9). However, deletion of this sequence (ALTKHFQD) has little effect on the net charge of fusion proteins P and N vs. P′ and N′, strongly suggesting that altered secondary/supersecondary structure is responsible for loss of DNA binding by the N and P proteins as well as the loss of antigenicity. The importance of protein folding for DNA binding is further underscored by the necessity of a renaturation step before DNA binds efficiently to p70 on Southwestern blots, and by the effect of mutations designed to destabilize the helix-turn-helix motif (Fig. 7). A similar approach has been used to investigate the effect of mutations in the POU-specific and POU homeo domains of Oct-1 (OTF-1) on DNA binding (26).

Analysis of the DNA binding properties of a series of p70 deletion mutants demonstrated that the minimal DNA binding site consists of aa 536–609. Structural integrity of p70 epitope 1 (aa 560–609) was necessary, but not sufficient, for DNA binding. Deletion of aa 601–609, or alteration of aa 591–593 (lys-ser-gly) to ala-ala-ala, eliminated both antigenicity and DNA binding (Figs. 4 and 7), suggesting that autoantibodies bind to p70 in a manner analogous to DNA, or that these changes destabilized the structure of aa 536–609 sufficiently to disrupt both autoantibody recognition and DNA binding. The strong dependence of both autoantibody and DNA binding on protein folding is consistent with the latter possibility, as is the observation that the autoantibodies react only weakly with aa 535–610 of murine p70 (10), whereas this sequence retains the ability to bind DNA (C.-H. Chou, unpublished observations). The simplest explanation is that the secondary or supersecondary structure of this region is critical for both autoantibody recognition and DNA binding, but that autoantibodies to epitope 1 might contact different amino acids from those contacting DNA. Nevertheless, we cannot at present rule out the alternative possibility that the same residues are involved in both autoantibody recognition and DNA binding.

The strongly basic domain adjacent to the potential helix-turn-helix also appears to be necessary, but not sufficient, for DNA binding. The importance of domains with high concentrations of basic amino acids in interacting with DNA has been underscored previously (29–31). Mutagenesis of this region to identify the critical residues for DNA binding is in progress. Finally, aa 506–535, although not required for DNA binding, appear to contribute to the efficiency of the p70-DNA interaction, in view of the stronger binding of DNA to fusion protein P′ then to fusion protein E on Southwestern blots and in the DNA immunoprecipitation assay (Fig. 5). This region also defines an autoepitope of p70 that is recognized by certain autoimmune sera, providing additional evidence that the DNA binding site of p70 may be preferentially targeted by autoantibodies.

In some respects, the p70 DNA binding site is reminiscent of the bipartite DNA binding sites of the octamer binding factors OTF-1 (Oct-1) and OTF-2 (Oct-2) (26, 32). Similarities in DNA sequence recognition by Ku or related antigens and octamer binding proteins have been noted previously (8). Like OTF-1 and OTF-2, p70 contains a potential helix-turn-helix motif (aa 585–603) that is involved in DNA recognition (Fig. 6). However, this region does not appear to be related to the homeo box consensus sequence found in Oct-1 (OTF-1), Oct-2 (OTF-2), and other proteins (32). In addition, the DNA binding domain of p70 does not contain sequences resembling the POU-specific domains shared by the Pit-1, OTF-1, OTF-2, and Unc-86 factors (33). Thus, although both p70 and the octamer binding proteins contain bipartite DNA binding domains, sequence comparisons do not strongly support the possibility of a functional relationship between these proteins. Although the present studies have not defined the function of Ku, identification of the DNA binding domain should permit this question to be addressed more readily.

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