Mechanism of Interaction between Ku Protein and DNA*

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The mechanism of interaction between the Ku autoantigenic protein, a heterodimer of noncovalently linked 70,000- and 80,000-dalton subunits, and DNA was studied using immunoaffinity-purified Ku protein and a 300-base pair EcoRI fragment from HeLa cell DNA. In the nitrocellulose filter-binding assay, the Ku protein bound 32P-labeled double-stranded DNA, and much less efficiently single-stranded DNA. The binding of Ku to DNA was dependent on ionic strength and prevented by IgG from patient sera containing anti-Ku antibodies. In competitive assays, using unlabeled nucleic acid competitors, the DNA binding of Ku was not inhibited in the presence of yeast tRNA, synthetic copolymer of poly(A)-poly(dT), or circular plasmid pBR322 DNA, but was inhibited when the plasmid DNA was cleaved with appropriate restriction endonucleases. The inhibitory activities of cleaved plasmid DNA were independent of the configuration or nucleotide sequences at ends but proportional to the number of recognition sites of restriction enzymes used. Footprint analysis demonstrated that K-1 protein protected both 3'- and 5' -terminal regions of double-stranded DNA from DNase I digestion. When Ku protein was fractionated electrophoretically, transferred to nitrocellulose filter, and probed with 32P-labeled DNA, only the 70,000-dalton subunit exhibited DNA binding. Thus, the Ku protein appears to recognize selectively ends of double-stranded DNA molecules. Possible functions of the Ku autoantigen in eukaryotic cells are discussed.

Autoantibodies against cellular constituents are found frequently in sera from patients with various autoimmune diseases. These antibodies are often useful to clinicians for diagnosis and evaluation of disease activity and to investigators as probes for studies of the structure and function of macromolecules (see reviews, see Refs. 1–3).

Recently, we described a new autoantibody termed anti-Ku which was found specifically in patients with scleroderma-polymyositis overlap syndrome (4). Initial studies of the Ku antigen demonstrated it to be a nonhistone DNA-binding protein heterodimer with 70,000- and 80,000-dalton (Da') subunits (5-7). This protein was accessible to antibody in dinucleosomal and larger segments of chromatin and thus appeared to be a component of chromatin.

We have now purified the Ku protein from HeLa cells using immunoaffinity chromatography and examined its interaction with DNA. These studies show that the Ku protein interacts specifically with the free end of linear double-stranded DNA.

MATERIALS AND METHODS

Sera—Ten sera with autoantibodies to the Ku antigen from Japanese patients with scleroderma-polymyositis overlap syndrome were used in this study. All sera were screened by Ouchterlony immunodiffusion, 32P immunoprecipitation, 35S immunoprecipitation, and immunoblotting to identify specificities of autoantibodies. IgG from these patient sera were purified using DEAE-cellulose (DE52, Whatman) equilibrated with 20 mM Tris-Cl, pH 7.2 (8).

Purification of Ku Protein—The Ku protein was purified from HeLa cell extract using immunoabsorbent column chromatography as described previously (5). The final eluate with 3.5 mM MgCl2 was dialyzed against Tris-buffered saline (TBS; 10 mM Tris-Cl, 150 mM NaCl, pH 7.5) and stored at -20°C in the presence of 50% glycerol until used. When 2 μg of this material (measured with Lowry assay) was fractionated on an SDS-10% polyacrylamide gel, only the 70,000- and 80,000-Da Ku polypeptides could be observed after staining with silver, and no DNA was detectable after staining with ethidium bromide (5).

DNA Probe for Filter Assay—High molecular weight nuclear DNA was extracted from HeLa cells according to the method of Gross-Bellard et al. (9). Fifty μg of this DNA in 10 μl of a buffer containing 50 mM Tris-Cl, pH 7.5, were digested with 50 units of EcoRI restriction endonuclease (Bethesda Research Laboratories) for 2 h at 37°C. The products were fractionated on a preparative 5% polyacrylamide gel, and a single 300-base pair (bp) band was excised and eluted with 0.5 M sodium acetate containing 1 mM EDTA. This procedure yielded 2 μg of a homogeneous-size DNA fragment. An aliquot was dephosphorylated with calf intestinal alkaline phosphatase and labeled at 5' ends with [γ-32P]ATP (Amersham Corp.) using T4 polynucleotide kinase (Bethesda Research Laboratories) as described by Maxam and Gilbert (10). Another aliquot was labeled at recessed 3' ends with [α-32P]dATP (Amersham Corp.) using the Klenow fragment of Escherichia coli DNA polymerase I (Boehringer Mannheim) (11).

Filter-binding Assay—The ability of the Ku protein to bind DNA was measured in the nitrocellulose filter assay described by Riggs et al. (12). Prior to use, nitrocellulose filters were soaked in 0.5 M KOH for 15 min and washed exhaustively with distilled water and subsequently with TBS. In the assay, 5 μg of the 300-bp DNA probe end labeled with 32P (25 fmol, approximately 10,000 cpn) were combined with various amounts of Ku protein in 400 μl of binding buffer A (10 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, and 50 μg/ml of bovine serum albumin, pH 7.5). The mixture was incubated at room temperature for 30 min and passed through one of the nitrocellulose filters described above. After three washes with 1 ml each of TBS, radioactivity retained on the filter was measured as Cerenkov radiation using the tritium channel of a liquid scintillation counter. In some experiments, the NaCl concentration of the binding buffer was varied between 0 and 0.5 M and adjusted to 0.15 M just

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3 The abbreviations used are: Da, dalton; bp, base pair; SDS, sodium dodecyl sulfate; DNase, deoxyribonuclease; TBS, Tris-buffered saline.
prior to passing the assay mixture over the nitrocellulose filter.

In an experiment to determine the effect of anti-Ku antibodies on the DNA-binding activity of Ku protein, 10 ng of Ku protein was incubated with various amounts of IgG purified from patient serum for 1 h at room temperature before the \[^{32}P\]DNA fragment was added.

In competitive assays designed to examine the ability of Ku to bind various DNA fragments or other nucleic acids, various amounts of unlabelled nucleic acid competitors were mixed with 5 ng of 300-bp \[^{32}P\]DNA in 400 \(\mu\)l of binding buffer A, and then 10 ng of Ku protein were added to the reaction mixture. The filter assay was performed as described above. Competitors included yeast tRNA (Sigma), sonicated salmon testis DNA (mean size 500 bp, Sigma), poly(A)-poly(dT) (mean size 3000 bp, Pharmacia P-L Biochemicals), a mixture of 5'-mononucleotides (dAMP, dGMP, dCMP, and dTMP, Sigma), and plasmid pBR322 DNA (4362 bp, mixture of form I and form II, Sigma). Additional competitors were derived from plasmid pBR322 DNA with various restriction endonucleases (EcoRI, HaeIII, HinfI, PstI, and PvuII, purchased from Bethesda Research Laboratories) according to published instructions (13). After digestion, each preparation was extracted with phenol-chloroform to remove protein and precipitated with ethanol.

**RESULTS**

**DNA-binding Properties of Ku Protein**—The ability of the Ku protein to bind to the 300-bp DNA probe was assessed in the nitrocellulose filter assay. In this assay free DNA passes through the filter while DNA bound to protein is retained. It can be seen that Ku binds double-stranded DNA more readily than single-stranded DNA. At maximal binding, 40 fmoles of Ku protein are capable of binding 90% of the double-stranded DNA probe (25 fmoles) used in the assay. The ability of Ku to bind DNA was dependent on the ionic strength of the binding buffer (Fig. 2). Binding was maximal at 0.05-0.20 M NaCl, was diminished slightly at 0 M NaCl, and was markedly reduced at NaCl concentrations greater than 0.35 M. Binding did not require Mg\(^{2+}\) and was not affected by temperature (0-37°C). Retention of some DNA at high salt probably occurs because of rapid reassociation of the DNA-Ku complex as the salt concentration is readjusted to 0.15 M just prior to passage of the solution over the filter (approximately 10 s elapse prior to actual filtration). When ionic strength is not readjusted no DNA is retained at high salt concentrations. When used alone, the naked DNA probe gave no binding to the filter.

IgG from patient sera known to contain anti-Ku antibodies inhibited the binding of Ku to DNA. Fig. 3 shows a typical study in which 100 \(\mu\)g of IgG inhibited binding by 95%. In these studies, the IgG fraction of all 10 anti-Ku sera produced...
In contrast, IgG from normal human serum or randomly little (one serum caused 41.4% inhibition) or no inhibition selected patient sera lacking anti-Ku antibodies produced significant inhibition (97.6 to 50.5% inhibition at 100 pg/ml). (Table I).

Tnating 5 ng (25 fmol) of 300-bp [32P]DNA and 10 ng (67 fmol) of Ku protein. As shown in Fig. 4, sonicated salmon testis tRNA were about 200-500-fold less effective (2 and 4 pg required, respectively, to produce 50% inhibition), while the mononucleotide (5'-dNMP) produced no inhibition at all. These results suggested that Ku binds selectively to DNA but may not recognize a specific base sequence.

To extend these observations further, we examined the binding of Ku to different forms of the DNA from plasmid pBR322 (Fig. 5, A and B). In its circular form (mixture of form I and form II) this plasmid DNA did not compete with the binding of Ku to the [32P]DNA probe. However, when the same DNA was denatured by heating or especially when cleaved with appropriate restriction endonucleases strong competitive inhibition was observed. As shown in Fig. 5A, 50% inhibition was achieved when 60 ng of heat-denatured plasmid DNA, 35 ng of EcoRI digest, 8 ng of HinfI digest, or 4 ng of HaeIII digest were added to the assay mixture. Since EcoRI, HinfI, and HaeIII have 1, 10, and 22 recognition sites, respectively, on pBR322 DNA (4362 bp) (16, 17), the inhibitory activity of these restriction fragments seems to be proportional to molar concentrations of DNA free ends.

Since EcoRI and HinfI yield DNA fragments with 5'-protruding ends of 4 and 3 nucleotides, respectively, the possibility existed that Ku recognized this particular DNA configuration. To test this possibility, we examined the ability of Ku to bind to different fragments of pBR322 DNA derived with restriction endonucleases that have equal numbers of recognition sites on this DNA but which yield different end constructions. Fig. 5B shows that pBR322 DNA fragments produced with three enzymes that are single cutters, EcoRI (5'-protruding end), PstI (3'-protruding end), or PvuII (blunt end) were approximately equal in ability to competitively inhibit the binding of Ku to the [32P]DNA probe. Similar results were obtained with pBR322 DNA digests prepared with HinfI (which has 10 recognition sites and yields 5'-protruding ends) and HaeIII (which has 11 sites and yields 3'-protruding ends) except that these latter enzymes were considerably more potent inhibitors as would be expected from the greater number of DNA fragments generated. Thus, the Ku protein appears to bind specifically to free ends portions of linear double-stranded DNA without regard to configuration or base sequence.

**Table I**

| Patients | Diagnosis* | Inhibition | DNA binding | Associated antibodies to |
|----------|------------|------------|-------------|-------------------------|
|          |            | %          | anti-DNA    |                         |
| 1        | PSS-PM     | 90.1       | 1.3         | Ku                      |
| 2        | PSS-PM     | 97.6       | 0.7         | Ku                      |
| 3        | PSS-PM     | 85.3       | 2.3         | Ku                      |
| 4        | PSS-PM     | 96.1       | 0.6         | Ku, Ro                  |
| 5        | PSS-PM     | 73.5       | 1.1         | Ku, U1RNP               |
| 6        | SLE-PSS-PM | 78.5       | 1.6         | Ku, Ro                  |
| 7        | SLE-PSS-PM | 50.5       | 7.1         | Ku, U1RNP, Ro           |
| 8        | SLE-PSS-PM | 93.8       | 9.1         | Ku, U1RNP, Sm           |
| 9        | SLE-PM     | 85.6       | 1.1         | Ku, Ro                  |
| 10       | SLE-PM     | 71.4       | 0.6         | Ku, Ro                  |
| 11       | SLE-PM     | 61.1       | 1.1         | Ro                      |
| 12       | SLE-PM     | 0          | 2.4         | La, Ro                  |
| 13       | SLE-PM     | 16.4       | 1.8         | Ro, Ki                  |
| 14       | SLE-PM     | 0          | 78.7        | Ribosome                |
| 15       | SLE-PM     | 41.4       | 3.5         | U1RNP, U3RNA            |
| 16       | SLE-PM     | 0          | 89.9        | U1RNP, Sm               |
| 17       | SLE-PM     | 0          | 1.9         | Sm, Ro                  |
| 18       | SLE-PM     | 0          | 17.4        | Ribosome                |
| 19       | SLE-PM     | 0          | 89.0        | Ro                      |
| 20       | SLE-PM     | 11.3       | 8.8         |                          |
| 21       | MCTD       | 11.5       | 1.4         | U1RNP                   |
| 22       | PSS-PM     | 12.4       | 1.5         | ScI70                   |
| 23       | PM-PM      | 28.8       | 1.0         | Jo-1                    |
| 24       | Normal     | 0          | 1.2         |                         |

*PSS, progressive systemic sclerosis (scleroderma); PM, polymyositis; SLE, systemic lupus erythematosus; MCTD, mixed connective tissue disease.

![Fig. 4. Competition of various nucleic acids or nucleotides with the 32P-labeled 300-bp DNA fragment for binding by Ku protein.](image)

**DISCUSSION**

The Ku antigen was initially described as an acidic nuclear protein that migrated in gel filtration with an apparent mo-
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FIG. 5. Competition assay performed with various forms of plasmid pBR322 DNA. Assay mixtures contained 5 ng of [\(^{32}\)P]DNA, 10 ng of Ku, and 0-200 ng of unlabeled pBR322 DNA in 400 \(\mu\)l of binding buffer A. A, circular pBR322 DNA (form I and form II) (O), or same DNA denatured by heating (C), or digested with EcoRI (\(\Delta\)), HindIII (\(\Delta\)), or HaeIII (*) were used as competitors. The number of recognition sites for each enzyme are shown in parentheses. B, similar competition assay was performed using intact circular DNA (O) and circular DNA digested with the single cutters EcoRI (\(\Delta\)), PstI (C), and PvuII (C), as well as HindIII (\(\Delta\), 10 recognition sites with 5' protruding ends) and HaeII (M, 11 recognition sites with 3' protruding ends).

FIG. 6. Nuclease footprinting of Ku protein-binding site on 300-bp DNA fragment. Ten ng of 300-bp double-stranded DNA uniquely labeled with \(^{32}\)P at 5' or 3' ends of the EcoRI site were used for footprinting analysis.

FIG. 7. Blot of Ku protein probed by \(^{32}\)P-labeled 300-bp DNA fragment. Two ng of purified Ku protein were fractionated on a 10% SDS-polyacrylamide gel and transferred electrophoretically to nitrocellulose paper (see Amido Black stain at left). The paper was incubated with native (double-stranded DNA) or heat-denatured (single-stranded DNA) \(^{32}\)P/DNA probe (see autoradiogram at right). Peptides of 70,000 and 80,000 Da from HeLa cells labeled with \(^{35}\)S]methionine (5-7). Moreover, these same polypeptides were also detected in immunoblots, but certain patient sera recognized one or the other of them preferentially (5, 7). Thus, the Ku protein seemed to form a complex with DNA, and it appeared to bear multiple autoantigenic epitopes.

In the present study we have used a 300-bp DNA fragment derived from HeLa cells by EcoRI restriction enzyme digestion and labeled with \(^{32}\)P to investigate the interaction of Ku protein with DNA. The data indicate that Ku binds directly to this DNA under physiologic conditions, exhibits little or no binding at high (>0.35 M NaCl) ionic strengths, binds weakly to single-stranded DNA, and not at all to RNA or...
DNA-RNA hybrids. Moreover, certain anti-Ku antisera inhibit the interaction of Ku protein with DNA possibly because they recognize an autoantigenic epitope whose location favors steric interference or because they induce a conformational change in the Ku protein.

Three lines of evidence indicate that the Ku protein binds specifically to end regions of double-stranded DNA. 1) Circular plasmid DNA (form I and II) does not inhibit the interaction between Ku and the [32P]DNA probe. 2) Plasmid DNA treated with different restriction enzymes competes with this probe in proportion to the number of recognition sites for these enzymes. 3) DNase I footprinting demonstrates that the Ku protein protects both 3' (for about 20 bases) and 5' (for about 30 bases) ends of the 300-bp DNA probe from enzymatic degradation. Ku binds these DNA regions whether or not they are staggered or blunt, and no particular nucleotide sequences appear to be recognized preferentially since binding occurs to a variety of DNA fragments constructed with different restriction enzymes. Scatchard analysis of the interaction of the Ku protein with DNA has demonstrated that approximately 2 mol of the protein are bound by a single mol of DNA (data not shown). Finally the binding appears to be independent of the terminal phosphate group since 5'-dephosphorylated DNA still binds to Ku (data not shown). In contrast, Ku protein bound to single-stranded DNA with lower affinity and did not protect any specific region of single-stranded DNA from DNase I attack.

The blotting studies shown in Fig. 7 indicate that only the 70,000-Da Ku subunit interacts with DNA. Presently, only limited information exists about how this component is related to the 80,000-Da subunit. Following their isolation using immunoaffinity chromatography, the two polypeptides can be separated in SDS-polyacrylamide gels under nonreducing action of the Ku protein with DNA has demonstrated that Ku protein bound to single-stranded DNA with lower affinity and did not protect any specific region of single-stranded DNA from DNase I attack.

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We are not aware of another eukaryotic cell protein that binds selectively to DNA ends. In prokaryotes an exonuclease inhibitor protein induced by the bacteriophage Mu-1 is thought to block the action of several exonucleases on double-stranded DNA by binding to the ends of DNA fragments (18). However, this protein has not been characterized, and it is not possible presently to compare it structurally with the Ku protein described here. Several viral and bacteriophage proteins known to be involved with DNA synthesis are found linked covalently to DNA ends (19–21). They include the adenoviral terminal protein (of 55,000 Da derived from an 80,000-Da precursor) that binds covalently to dCMP and the P3 protein of the Bacillus subtilis phage Φ29 that binds covalently to dAMP. These protein-nucleotide complexes constitute initiation sites for replication of new DNA strands. Ku appears to be unrelated to these proteins because it does not recognize a specific terminal nucleotide and does not bind covalently to DNA.

Although direct proof is yet lacking, we infer that Ku protein binds DNA ends in vivo. Previous calculations indicate that on average each log phase HeLa cell contains about 4 x 10^6 copies of the Ku protein complex. It seems likely to us that this protein exists within the nucleus largely free and becomes associated with DNA break points that might occur through the action of such agents as UV light or certain drugs. This idea is consistent with the results of indirect immunofluorescence which indicate a nuclear location for the Ku antigen during interphase but no association with metaphase chromosomes (5). Similarly in cell extracts, Ku protein might bind to DNA fragments created when substrate cells are disrupted through sonication. This concept suggests that Ku protein plays a role in DNA repair or transposition.

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