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ON THE MECHANISMS OF KU PROTEIN BINDING TO DNA

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The *in vitro* DNA-binding activity of Ku protein, a heterodimer of 70 and 86 kDa subunits, was studied using affinity-purified protein. Ku protein bound to different DNA probes and displayed a multiple-band pattern in band mobility shift assays. The protein-DNA complex formation was effectively blocked by different DNA competitors, indicating a non-sequence specific binding of Ku protein to DNA; no preference of binding of Ku protein to regulatory sequences derived from U1 snRNA, U6 snRNA or nucleolar protein p120 genes was observed. The number and size of the Ku protein-DNA complexes increased with increasing of the protein concentration and the size of DNA probe, suggesting that the protein accumulates on the DNA fragment until saturation of the binding sites. In UV-crosslinking experiments, the binding of Ku protein to DNA was shown to start with the 70 kDa subunit contacting free DNA ends.

The Ku protein is a heterodimer composed of two subunits: 70 kDa and 86 kDa, which was originally detected as an autoantigen reacting with antibodies from patients with rheumatic disorders (1). The localization of this protein complex to the DNase sensitive regions in the chromatin was the first indication that the Ku protein may be involved in active DNA processes such as transcription, replication, or repair (2). Recently, it was reported that the Ku protein is involved in specific binding to the promoter region of human transferrin receptor gene (3) and to the proximal and distal promoter elements of the U1 snRNA gene (4). In addition, the *in vitro* transcription of U1 snRNA gene depended to a large extent on the presence of the Ku protein (4, 5).

Based on analysis involving protein denaturation (Southwestern blots), it was proposed that *in vitro* Ku protein binds to free DNA ends through the 70 kDa subunit (6, 8). In the present study, using UV-crosslinking we showed that the 70 kDa subunit directly contacted DNA ends in the native protein complex. No sequence specificity of the DNA-binding activity was observed when the affinity-purified Ku protein complex was reacted with different DNA fragments as probes or competitors in band mobility shift assays.

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Abbreviations: DTT, dithiothreitol; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl polyacrylamide gel eletrophoresis.

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MATERIALS AND METHODS

Purification of Ku protein. The Ku protein was extracted from HeLa cells and purified by DEAE-cellulose and phosphocellulose chromatography as previously described (9). The final step of purification involved either immuno-affinity (9) or DNA-affinity chromatography. DNA-affinity column was constructed using concatenated 45-bp oligonucleotides from the 5' upstream region of the p120 nucleolar protein gene (10). All solutions used in the purification contained 5 mM DTT.

DNA fragments and labeling. A 125-bp fragment (F1) was prepared from a pTZ18R subclone of the 5' flanking region (-1430/-1327) of the p120 gene (10). A DNA fragment containing proximal sequence element of U1 snRNA gene between -7 and -112 nucleotides was released by digestion of pHU1-ID (11) with Pvu II and Bgl II restriction enzymes. A DNA fragment from the mouse U6 snRNA gene promoter (-315/1) was generated by digestion of a plasmid with Dra I and Eco RI enzymes (12). All DNA fragments were purified from agarose gel slices and end-labeled with Klenow enzyme or uniformly-labeled by PCR in the presence of [α-^{32}P]dATP (13).

Band mobility shift assay. In 20 µl reactions, ^{32}P-labeled DNA fragments (1 ng) were mixed with various amounts of purified Ku protein as indicated in the figures in a buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 0.1 mM EDTA, 0.25 mM DTT, 200 mM KCl and 7% glycerol. After incubation at room temperature for 20 min, the protein-DNA complexes were analyzed on 5% polyacrylamide gels as described (14). For blocking experiments the protein was first incubated with increasing amounts of cold DNA competitors for 30 min at room temperature and then allowed to bind to ^{32}P-labeled DNA probes.

UV-crosslinking experiments. The purified Ku protein was mixed with the F1 fragment either end-labeled with Klenow fragment or uniformly-labeled by PCR in a 1:4 DNA/protein molar ratio in 30 µl final volume containing 20 mM Tris-HCl (pH 7.5), 200 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.25 mM DTT, and 7% glycerol. After 15 min incubation at room temperature the complexes were irradiated with a short-wavelength (15W) UV lamp at 4°C for 20 min. This was followed by adding 45 µl of nuclease digestion buffer: 20 mM Tris·Cl (pH 8.0), 1% NP-40, 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 1 µl of 5 U/µl DNase I and 1 µl of 5 U/µl micrococcal nuclease as described previously (15). After 30 min digestion at 37°C, the reactions were terminated by adding 25 µl of 4x SDS-loading buffer, boiled for 5 min and analyzed on 10% SDS-PAGE (16). Autoradiograms were prepared by exposing dried gels to XAR-5 film with intensifying screen at -70 °C for 20 h.

RESULTS

The affinity-purified Ku protein binds to DNA. The Ku protein purified either by DNA-affinity or immuno-affinity chromatography formed identical complexes with DNA probe (F1) as detected by band mobility shift assay (Fig. 1). The relative amounts of the four major and two minor bands depended on the protein concentration, suggesting that the slow-migrating complexes are derived from the faster-migrating complexes. Preincubation of Ku protein with either autoantibodies or monoclonal antibodies against Ku protein resulted in a significant decrease of electrophoretic mobility of the Ku-DNA complexes (Fig. 2, lanes 2-4). These results showed that it was the Ku protein that formed complexes with DNA probe and the antibodies bound to Ku protein-DNA complexes did not affect the DNA-binding activity of the protein. No effect on DNA binding was detected after preincubation of Ku protein with a non-related monoclonal antibody specific for a 250-kDa membrane antigen of HeLa cells (Freeman, unpublished results) (Fig. 2, lane 6).

DNA-binding specificity of the affinity-purified Ku protein. Previously, Ku protein was shown to bind specifically to the proximal sequence element (PSE) of U1 snRNA gene in DNase I footprinting (4). To determine the DNA-binding specificity of the affinity-purified Ku

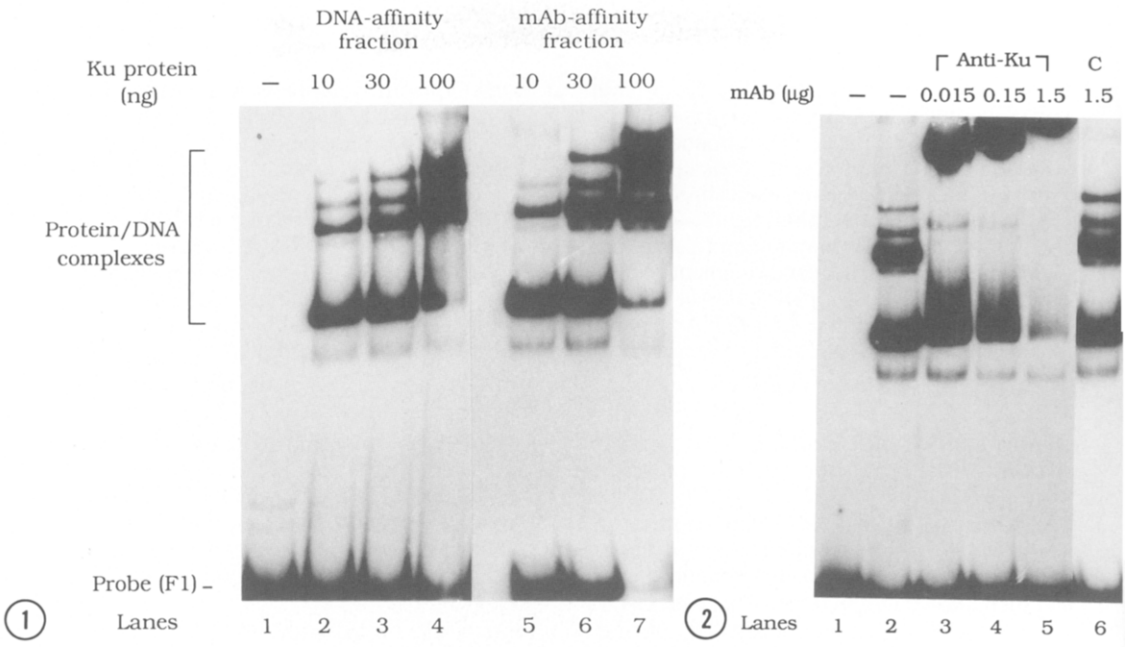

Fig. 1. DNA binding of affinity-purified Ku protein. Various amounts of Ku protein purified by DNA-affinity (lanes 2-4) or immuno-affinity (lanes 5-7) chromatographies were incubated with 1 ng ^{32}P-labeled DNA probe (F1 fragment) for 15 min at room temperature. The electrophoretic mobility of the protein-DNA complexes were analyzed in 5% polyacrylamide gel as described in "Materials and Methods".

Fig. 2. Effect of monoclonal antibodies on Ku protein DNA-binding activity. Purified Ku protein (10 ng) was preincubated with various amounts of antibodies for 30 min at room temperature and then allowed to bind to ^{32}P-DNA probe (F1). The migration of Ku-DNA complexes was analyzed in 5% polyacrylamide gel. Lanes 3-5, monoclonal antibody D_6D_8 (mAb) specific for the 86 kDa subunit of Ku protein (19). Lane 6, a negative control (C) using a monoclonal antibody against HeLa membrane protein (Freeman, unpublished results).

protein, competition analyses were carried out in band shift assays using U1 PSE and a DNA fragment from the region (-315/ 1) of the U6 snRNA gene, which was shown to be a functional analog to the PSE of U1 snRNA gene (12). As shown in Fig. 3, all of the DNA competitors including unlabeled F1 fragment and poly dI-dC, effectively blocked the DNA-binding of Ku protein under the same DNA/protein concentrations. When ^{32}P-labeled U1 snRNA DNA fragment was used as a probe in similar blocking experiments, the DNA binding of Ku protein was again effectively abolished by all DNA competitors including cold U1, U6 snRNAs, F1, and poly dI-dC (data not shown). These results demonstrated that *in vitro* the purified Ku protein bound to DNA without significant preference for particular sequences, but rather depended on the mass ratio of the protein to DNA.

Accumulative binding of Ku protein to DNA fragment. To examine further the binding properties of Ku protein to DNA, a non-related DNA fragment of different size from the -727/-282 region of the p120 gene was used as a probe. Ku protein bound to this probe, again displaying a similar multiple-band pattern (Fig. 4). Since this probe (445 bp) is longer than F1 fragment (125 bp), more retarded bands were seen in band shift assays. The size of the Ku-DNA complexes

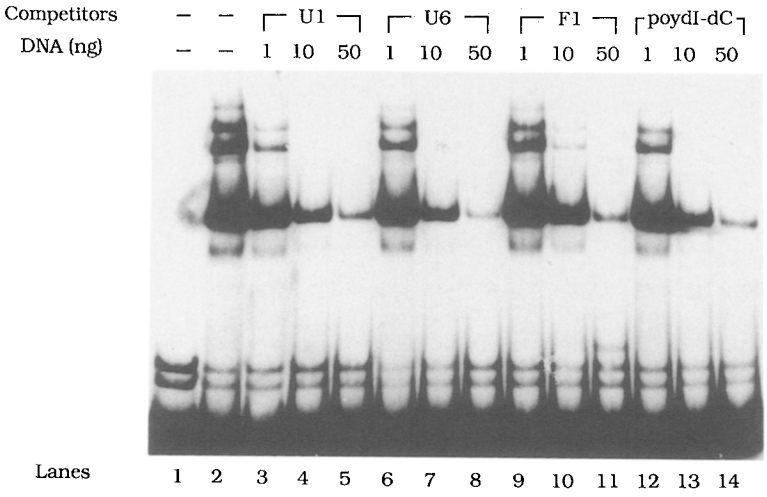

Fig. 3. Blocking of Ku protein DNA binding by different DNA competitors. The Ku protein was first incubated for 30 min with increasing concentrations of U1 (lanes 3-5), U6 (lanes 6-8), unlabeled F1 (lanes 9-11), and poly dI-dC (lanes 12-14) DNA fragments as indicated above each lane and then allowed to bind to ^{32}P-labeled F1 DNA fragment for 15 min at room temperature. The protein-DNA complexes were analyzed in 5% polyacrylamide gel.

increased with increasing the amount of Ku when the amount of the probe was constant. These results suggest that the Ku protein binds to DNA in an accumulative manner.

The 70 kDa subunit of Ku protein binds to free ends of DNA. Purified Ku protein was bound to DNA and the complex was covalently cross-linked by UV irradiation. When double-

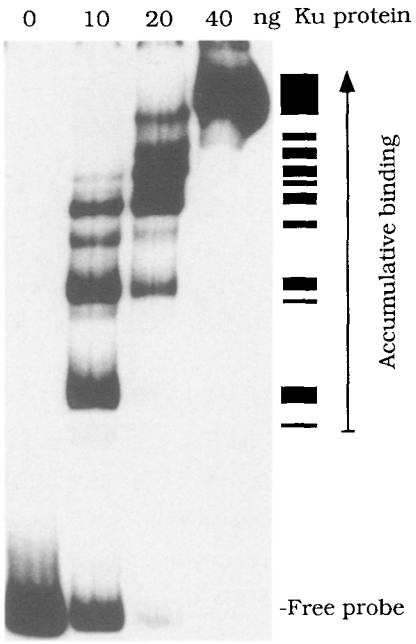

Fig. 4. Accumulative binding of Ku protein to DNA. Various amounts of the purified Ku protein indicated above each lane were incubated with 1 ng ^{32}P-labeled DNA probe (445 bp) for 15 min at room temperature, followed by electrophoretic analysis of the protein-DNA complexes in 5% polyacrylamide gel.

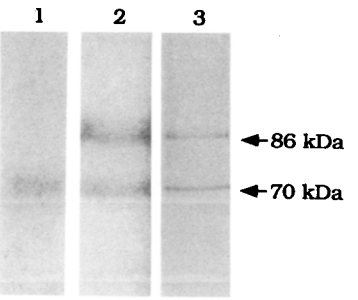

Fig. 5. UV crosslinking of the Ku protein to end-labeled and uniformly labeled DNA probes. The conditions for complex formation and UV irradiation are as described in "Materials and Methods". After nuclease digestion, the ^{32}P-tagged proteins were analyzed in 7.5% SDS-PAGE. The gels were stained with silver, then dried for autoradiography. Lane 1, autoradiography of Ku protein binding to end-labeled F1 fragment used as a probe. Lane 2, autoradiography of Ku protein binding to uniformly-labeled F1 probe. Lane 3, silver staining of Ku protein.

stranded DNA fragment labeled at the ends was used for binding, only 70 kDa subunit was tagged with radioactivity (Fig. 5, lane 1). When uniformly labeled DNA fragment was used, both subunits were labeled (Fig. 5, lane 2) as previously reported (4). In both cases the binding depended on the salt concentration with an optimum at about 200 mM KCl. These results showed that it was the 70 kDa subunit of the native Ku protein that bound to the free ends of DNA.

DISCUSSION

Affinity purified Ku protein bound to DNA, forming four major and two minor bands in electrophoretic mobility shift assays. A similar pattern of retarded bands was observed when purified Ku protein was bound to the adenovirus type 2 origin of replication (7). The effect of antibodies specific for Ku protein demonstrated that these bands were due to the binding of Ku protein to DNA. The multiple forms of the retarded bands are consistent with the idea that the protein bound first to the ends of DNA fragment and then slided along DNA to offer free ends for next Ku protein binding as previously reported (7). Therefore, the Ku protein binds to DNA by adding molecules at two ends of DNA until the fragment is completely covered with the protein molecules (accumulative binding). This was demonstrated by the band mobility shift assays in which the longer DNA probe (445 bp) produced more bands (Fig. 4) than the short probe (125 bp) did (Figures 2 and 3) and the size of the Ku-DNA complexes increased with increasing the amount of Ku when the amount of the probe remained constant (Fig. 4).

Previously, it has been reported that the 70 kDa subunit of Ku protein can bind to DNA in Southwestern blot analysis (6, 8). Here, using UV-crosslinking of the protein to end-labeled DNA fragment, we demonstrated that the 70 kDa but not 86 kDa subunit of the native Ku protein bound to the free DNA ends. Since it has been well documented that the purified protein did not bind to circular DNA (6, 7), the ^{32}P-labeling of 86 kDa subunit with uniformly labeled DNA probe, observed in this study and by others (3, 4), may reflect the ability of 86 kDa subunit to contact DNA as a consequence of the initial binding of the 70 kDa subunit to free DNA ends.

In vitro DNA binding experiments using different DNA fragments for blocking of the DNA-binding site did not reveal preference of the affinity-purified Ku protein for any particular

sequences. The previous reports of sequence-specific binding of Ku protein utilized mainly chemical and exonuclease DNA footprinting assays without presence of any DNA competitors (3, 4, 6, 7). Since high protein DNA ratios and short DNA fragments were used in these studies, the DNA footprints may represent Ku occupancy on the DNA probe with regular spaces in between rather than a sequences-specific protection. In a study on DNA binding of Ku protein to adenoviral DNA no significant sequence specificity of DNA protection pattern was observed with Ku protein alone (7). In our experiments, when the purified Ku protein was used in DNase I footprint experiments, similar regularly-spaced protection patterns were seen with different DNA probes (data not shown) as previously reported (4, 7, 17). The entry-sliding model of the protein-DNA interaction also indicates that *in vitro* Ku protein binds to DNA non-specifically. However, this may not be relevant to the *in vivo* situation where the protein may be positioned on specific DNA sequences depending on the presence of other DNA-binding proteins. Finally, *in vitro* DNA-binding sequence specificity may be demonstrated if more refined DNA binding techniques are used as those described for the c-*myc* protein for example (18).

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