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Analysis of the mechanism of interaction of simian Ku protein with DNA

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ABSTRACT

Ku protein is a relatively abundant DNA-binding protein which was first detected as the autoantigen in a patient with scleroderma-polymyositis overlap syndrome (hence the name 'Ku'). It is a heterodimer of two polypeptide chains of molecular weights 85 000 and 72 000, and it characteristically binds, *in vitro*, to the ends of DNA fragments, and translocates to form regular multimeric complexes, with one protein bound per 30 bp of DNA. We have studied the mechanism of interaction of Ku protein with DNA *in vitro*, using protein extracted from cultured monkey cells. We find that the precise structure of the DNA ends is not important for binding, as Ku protein can bind to hairpin loops and to mononucleosomes. Bound protein also does not require DNA ends for continued binding, since complexes formed with linear DNAs can be circularized by DNA ligase. Dissociation of the complex also appears to require DNA ends, since ligase closed circular complexes were found to be extremely stable even in the presence of 2 M NaCl. We also found that Ku molecules slide along DNA, with no preferential binding to specific sequences. Thus, Ku protein behaves like a bead threaded on a DNA string, a binding mechanism which allows us to make a new hypothesis concerning the function of this protein in the nucleus.

INTRODUCTION

Antibodies found in the sera of patients with autoimmune diseases have been invaluable tools for the study of nuclear proteins (for a review on antinuclear autoantibodies, see ref. 1). Among such proteins, Ku protein was initially identified in scleroderma-polymyositis overlap syndrome, 'Ku' being derived from the first patient's name (2). It was studied originally for its medical implications (3–8) and more recently has been of interest because of its possible importance in nuclear function. Ku protein has been detected by several different techniques and denoted by various names including NFIV (9), TREF (10), PSE1 (11), and Ku-2 (12).

Ku protein is relatively abundant, as it is present in about 4×105 copies per HeLa nucleus during exponential growth (5), and it is now known to be conserved among mammal species (8). The protein is a heterodimer composed of two polypeptide chains of molecular weights 85 000 and 72 000. The cDNAs coding for both protein subunits have been cloned and sequenced (13–17), and the deduced amino acid sequences suggest that the subunits might be held together by an element similar to a leucine zipper (18).

In contrast to this precise knowledge of Ku protein's structure, the role it plays in the nucleus remains unclear. First studies of the mechanism of interaction of Ku protein with DNA *in vitro* showed that the protein binds to ends of DNA fragments (6,9), and that, when additional molecules bind, the protein translocates along the DNA to form a regular DNA-multimeric protein complex (9). This binding mechanism suggested a possible role for Ku protein in repair of double strand breaks or in transposition (6,9). More recently, groups studying transferrin receptor and U1 RNA genes have detected what appears to be Ku protein, and have suggested that it interacts with specific sites in the promoters to play a role in transcription of both of these genes (10,11). Finally, protein Ku-2 has been found to give footprints on the octamer regulatory DNA motif, which is present in several transcriptional enhancer elements (12).

We have surveyed nuclear proteins of cultured monkey CV1 cells for binding, *in vitro*, to DNA of simian virus 40 (SV40). Among such proteins, one relatively abundant protein was found to show a strong affinity for probe DNA and initial studies of its binding mechanism suggested that it might represent the simian homolog of human Ku protein. We have confirmed this suggestion by demonstrating that anti-Ku autoantibodies recognize the subunits of the protein. Here we present further analysis of the mechanism of interaction of simian Ku protein with DNA, the results suggesting a new functional role for Ku protein *in vivo*.

MATERIALS AND METHODS

DNA fragments

Unless otherwise indicated, labeled DNA fragments used for binding were either the 172 base pair (bp) *Hin*dIII monomer of satellite DNA from cultured African green monkey CV1 cells (19), or the 224 bp *Sty*I fragment from the cloned control region of SV40, extending from map positions 37 to 333, with one of the 72 bp repeats in the transcription enhancer deleted (20). They were 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase.

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DNA circles were prepared from the 224 bp *Sty*I fragment by reaction with T4 DNA ligase in the presence of increasing amounts of ethidium bromide. Upon removal of ethidium bromide, supercoiled circles were fractionated by electrophoresis on a 4% polyacrylamide gel of the type used in gel retardation experiments. Nicked circles were purified in two steps, because they showed the same mobility as relaxed covalently closed circles and linear trimers. Covalently closed circles with one negative supercoil were first purified by electrophoresis and electroelution. These circles were digested with DNase I and resubmitted to gel electrophoresis, and nicked circles were then electroeluted.

A 360 bp DNA molecule with hairpin loops at both ends was prepared from a *Hin*dIII-*Hpa*II fragment from SV40 DNA (map positions 5171−346, one of the 72 bp repeats deleted). It was first treated with T4 DNA ligase, and the ligation products were digested with *Hin*dIII, yielding mostly head-to-head dimers of the initial fragment with *Hin*dIII sites at both ends and one *Hpa*II site in the middle. These dimers were heat-denatured and allowed to reanneal, yielding monomers with a hairpin loop at the *Hpa*II site. The molecules were then dephosphorylated, gel purified, 5′ end labeled, and a hairpin loop was added to the *Hin*dIII end by ligation of a *Hin*dIII 'splinker' from Boehringer, a 28-base synthetic oligonucleotide formed of a hairpin loop terminated by a cohesive *Hin*dIII end. After final gel purification, both phosphatase and exonuclease III treatment confirmed that the molecules possessed no free ends or single strand nicks.

Nucleosomes extracted from CV1 nuclei by digestion with micrococcal nuclease were labeled with [γ-^{32}P]ATP and polynucleotide kinase. They were fractionated by electrophoresis on a preparative retardation gel, and H1-depleted mononucleosomes were electroeluted as described (21).

Unless otherwise indicated, non radioactive competitor DNA was *E. coli* DNA, sonicated to an average size of 1 kbp. Synthetic homopolymers were from Pharmacia.

Purification of Ku protein

HPLC fractions containing Ku protein were prepared simultaneously with the sequence-specific single strand DNA-binding protein H16, according to procedures described elsewhere (21), and were generously given to us by Claire Gaillard. Briefly, nuclei were purified from cultured monkey CV1 cells, proteins were extracted with 0.4 M NaCl, and fractions were obtained by chromatography on hydroxyapatite. Fractions which contained protein H16 also contained Ku protein and were pooled, dialyzed, and submitted to HPLC using a mono Q column (Pharmacia). Elution from mono Q was performed with a linear gradient of NaCl. Ku protein eluted as a sharp peak at 0.24 M NaCl, well after H16. Ku protein fraction appeared to be pure with respect to DNA-binding activity but still contained many other proteins as can be judged from Fig. 2A.

Gel retardation electrophoresis

Protein was incubated with DNA for 15 mn at 37°C in 25 μl of 100 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 100 μg bovine serum albumin/ml, 0.1% Triton X100. Complexes were analyzed by gel retardation on a 4% polyacrylamide gel in 6.7 mM Tris-HCl, 3.3 mM Na Acetate, 1 mM EDTA, pH7.6, as described previously (22). When gel retardation was performed on a mixture of different labeled DNA fragments, it was often difficult to distinguish the different complexes, due to the large number of bands. In such cases, electrophoresis was performed in a second dimension in a polyacrylamide gel containing SDS as described (21). Preparative gel retardation for further analysis of bound protein by SDS-polyacrylamide electrophoresis was performed as described (21).

Circularization of Ku protein-DNA complexes

A labeled 224 bp *Sty*I fragment (10 000 cpm) was incubated with decreasing amounts of Ku protein in 25 μl of 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 0.1% triton X100, 100 μg bovine serum albumin/ml. After 10 mn at 37°C, 40 units of T4 DNA ligase were added and incubation continued for 1 hr. As ligase also bound to DNA under the conditions used, 300 ng of bacteriophage lambda DNA were then added and incubation continued for 10 mn, allowing the ligase to redistribute on the non-radioactive competitor, and taking advantage of the stability of the Ku protein-DNA complex (9). The complexes were then directly analyzed on a retardation gel.

Immunoblotting

After electrophoresis, proteins were transferred to nitrocellulose following standard procedures. The membranes were then incubated with sera from patients with anti-Ku autoantibodies, kind gifts from T. Mimori, W.H. Reeves, and E.M. Tan. Bound antibodies were detected by incubation with secondary antibodies directed against human IgG coupled to alkaline phosphatase, followed by detection of alkaline phosphatase by appearance of dark purple color in the presence of a chromogenous substrate (ProtoBlot from Promega).

RESULTS

Cultured monkey cells contain a protein homologous to human Ku protein

While fractionating nuclear extracts from cultured monkey CV1 cells to purify sequence-specific DNA binding proteins for the control region of SV40, we detected a protein which possessed

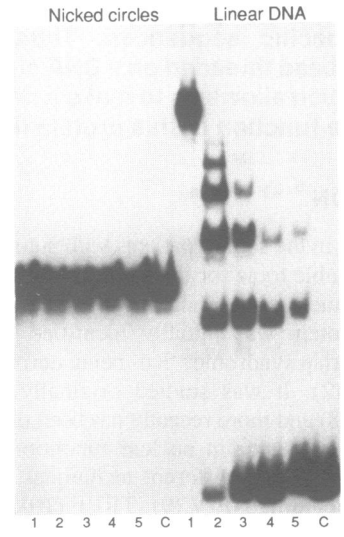

Figure 1. Interaction of monkey Ku protein with DNA minicircles. Nicked DNA circles formed with the labeled 224 bp DNA fragment were incubated in the presence of monkey protein and analyzed by gel retardation. The same experiment was performed in parallel with the linear DNA fragment. Competitor DNA amounts: 0, 4, 8, 15, 30 ng in lanes 1−5, respectively. Lanes C: control, no protein added.

DNA-binding characteristics similar to those already published for human Ku protein (6,9). First, in gel retardation experiments, the monkey protein formed a ladder of multimeric complexes with any of several different DNA fragments (see Fig. 1). Second, the maximum number of ladder rungs, i.e. the maximum number of protein molecules bound per fragment, increased as a linear function of the fragment sizes, with an average of one protein molecule per 30 bp of DNA (9). Third, a given DNA interacted differently with the protein depending on its fragment size: for example, intact circular plasmid pBR322 was a poor competitor for binding, but became a good competitor when linearized, and a much better one when cut into 22 fragments with HaeIII (6,9). Fourth, the monkey protein did not bind to DNA minicircles (see Fig. 1). Fifth, the protein showed no redistribution when bound to a given DNA fragment, even when challenged with a large amount of competitor DNA and after an extended incubation time (9). These data (not shown) were the same as results which led others to the conclusion that human Ku protein binds primarily to the ends of DNA fragments (6,9), that additional protein molecules can then bind to the fragment ends and translocate along DNA, forming regular DNA-multimeric protein complexes (9), and that once bound to a given DNA fragment the protein cannot detach easily to move to another fragment (9).

The molecular weight of the monkey protein was determined by binding partially purified protein to DNA and then purifting the complex by preparative gel retardation (21). Protein in the complex was analyzed by electrophoresis on an SDS-polyacrylamide gel with silver staining (Figure 2A). It was found to contain two stoichiometric polypeptide chains of apparent molecular weights 85 000 and 72 000, as does human Ku protein (3–5,9).

To confirm the homology further, we used sera from four patients with autoimmune diseases, which were kindly sent to us by T. Mimori, W.H. Reeves and E.M. Tan. All gave very similar results. Figure 2B shows an immunoblot with one of our preparations of monkey protein (lane 1) and a total protein extract from HeLa cells (lane 2), both showing identical positive bands. In addition, a retardation gel containing complexes of our protein with a labeled DNA fragment was transferred to nitrocellulose and probed with anti-Ku serum (this serum contained no anti-DNA antibodies, data not shown). Figure 2C shows an autoradiogram of the gel before transfer, and Figure 2D the immunoblot. All complexes in the ladder are recognized by the anti-Ku serum, indicating further that the protein from monkey studied here is in fact the homolog of human Ku protein.

The following experiments were performed to further study the mechanism of interaction of Ku protein with DNA.

Ku protein can remain bound to a circular DNA molecule

If DNA ends were absolutely necessary to the Ku protein-DNA complex, it should not be possible to circularize a DNA fragment to which Ku protein was already bound, or Ku protein should be released during the ligation. Figure 3 shows, however, that it is possible to form such structures. Variable amounts of Ku protein were bound to a labeled DNA fragment with cohesive ends. The complexes formed were analyzed by gel retardation, either directly (Fig. 3A), or after incubation in the presence of T4 DNA ligase (Fig. 3B). The DNA of the ligated complexes was also analyzed on the same type of gel after dissociation of the protein by addition of SDS (Fig. 3C). It is observed that the presence of Ku protein does not inhibit DNA ligase but that, at a high protein/DNA ratio, the formation of circular DNA molecules is inhibited whereas the formation of linear oligomers

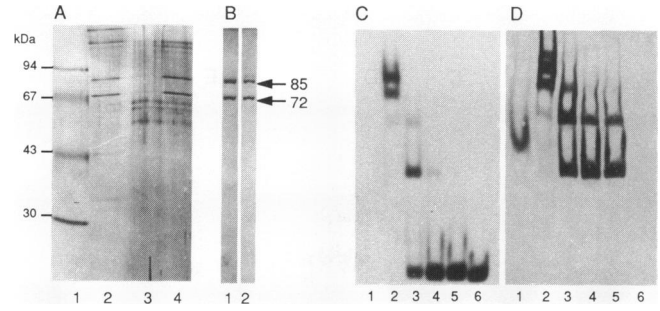

Figure 2. Homology between monkey protein and human Ku protein. (A) Monkey protein studied by SDS-polyacrylamide electrophoresis. The complex formed by the monkey protein with 100 ng of a 172 bp DNA fragment was cut from a preparative retardation gel and analyzed by electrophoresis on an SDS-polyacrylamide gel with silver staining. Lane 1: molecular weight marker; lane 2: protein fraction used for complex formation; lane 3: control, protein without DNA was loaded on the retardation gel; lane 4: complex between Ku protein and DNA, the DNA migrated to the gel bottom. (B) Immunoblotting comparison between the monkey protein and human Ku protein. A fraction containing the monkey protein (lane 1) and a total nuclear extract from HeLa cells (lane 2) were loaded on an SDS-polyacrylamide gel and analyzed by immunoblotting with serum from a patient with anti-Ku autoantibodies. (C) and (D) Immunoblotting analysis of the complexes between the monkey protein and DNA. In (C), complexes between the monkey protein and a labeled 172 bp DNA fragment were analyzed by gel retardation and autoradiography. Lane 1: free protein, no DNA added; lanes 2–5: fixed amount of protein with increasing amounts of non-radioactive 172 bp DNA fragment, plus a constant amount of labelled DNA as a tracer; lane 6: free DNA, no protein added. In (D), after autoradiography, the polyacrylamide gel shown in (C) was analyzed by immunoblotting with a human serum containing anti-Ku autoantibodies.

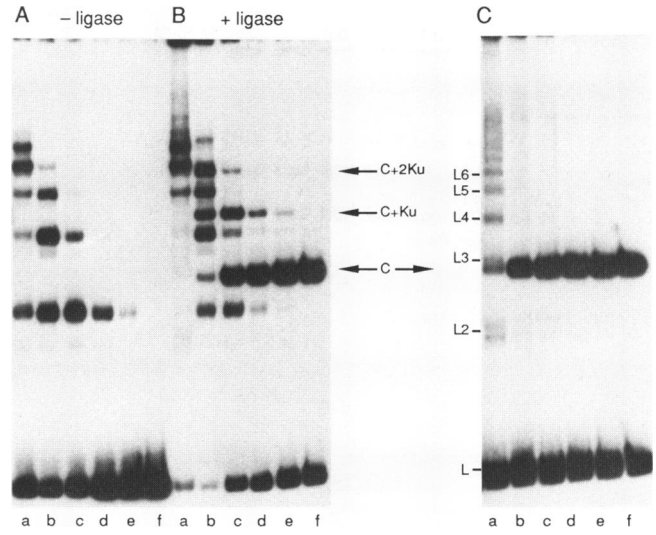

Figure 3. Circularization of DNA fragments with Ku protein bound. A labeled 224 bp DNA fragment with 4 bp cohesive ends was incubated with threefold serial dilutions of Ku protein in the absence of any competitor DNA. The complexes were then either directly analyzed on a retardation gel (A) or incubated with T4 DNA ligase before loading on the gel (B). In (C), after ligation, DNA was dissociated from protein by addition of SDS prior to electrophoresis. L indicates the position of the linear DNA, L2-L6 the positions of the linear oligomers of the DNA fragment, C indicates monomeric circles, a mixture of nicked circles and relaxed covalently closed circles, C+Ku and C+2Ku indicates the complexes of monomeric DNA circles with 1 and 2 molecules of Ku protein, respectively.

is favored (panel C, lane a, compare to lanes b−e; also note that at the DNA concentration used, only monomeric circles are formed when no protein is added: panel C, lane f). This suggests that Ku protein rigidifies the portion of the DNA segment to which it is bound. Analysis of the complexes after incubation with DNA ligase shows, in addition to the usual bands corresponding to complexes with linear DNA molecules, some extra bands (arrows) that correspond to monomeric DNA circles with 0, 1, 2, and sometimes 3 molecules of Ku protein bound. The identity of the DNA in these bands was confirmed by electroelution and gel electrophoresis: the bands noted C+Ku and C+2Ku contain monomeric circles in the proportion of about 25% nicked circles and 75% relaxed, covalently closed circles (data not shown). The presence of Ku protein in these bands was also confirmed by immunoblotting (data not shown). These results show that, although Ku protein needs DNA ends for binding, it can however remain bound to a DNA molecule with no ends.

Complexes of Ku protein and DNA circles are resistant to high salt concentrations

The influence of DNA ends in the mechanism of interaction of Ku protein with DNA is further studied in the experiment shown in Figure 4, where the redistribution of the protein bound to either circular or linear DNA is studied as a function of the salt concentration. After incubating the complexes with T4 DNA ligase as in Figure 3, a large excess of non-radioactive competitor DNA was added, together with NaCl to the indicated concentrations, from 0.1 M to 2 M. After incubation at 37°C, the NaCl concentration was lowered by dilution, and the samples were analyzed by gel retardation. No redistribution is observed in 0.1 M NaCl, confirming a known property of Ku protein (9). At the NaCl concentrations from 0.35 to 2 M, the complexes of Ku protein with linear DNA are dissociated, whereas the complexes with DNA circles remain stable, even in 2 M NaCl.

Ku protein can recognize hairpin loops in DNA

Ku protein binds indifferently to DNA ends whether 5′ protruding, blunt, or 5′ recessed, phosphorylated or not, and can also bind to linear or circular single-stranded DNA (6,9). It has also been suggested that the opening of the DNA double helix at the ends of the fragments might play a role in Ku protein binding (9). The experiment shown in Figure 5 brings some information as to what in a DNA end is recognized by the protein. On one hand, E. coli denatured DNA is almost as good a competitor as native DNA (Fig. 5A,B). But, on the other hand, the synthetic homopolymers poly dA, poly dT, poly dG, and poly dC, that cannot form B-type double helical DNA, do not compete at all for binding (Fig. 5C), which suggests that the protein recognizes some particular secondary structures of the single strands of E. coli DNA. An extreme example is shown using as a labeled substrate a DNA fragment to which hairpin loops were ligated at both ends, thus forming a double-stranded linear molecule that is also a single-stranded circle (Fig. 5D). This substrate is recognized by Ku protein just as well as a linear DNA fragment with regular ends. This argues against the possible role of double helix opening in Ku protein binding to a DNA end, but suggests that, in single strand, palindromic secondary structures are actually recognized. In agreement with this interpretation, synthetic poly (dIdC) is an excellent competitor (not shown). Finally, using H1-depleted mononucleosomes as a substrate, we were able to bind a maximum of two molecules of Ku protein per nucleosome (Fig. 5E), which suggests that Ku protein can still bind to the ends of DNA but cannot translocate when histones are present. We should also mention that, although Ku protein appears to be bound to DNA *in vivo* (see Discussion),

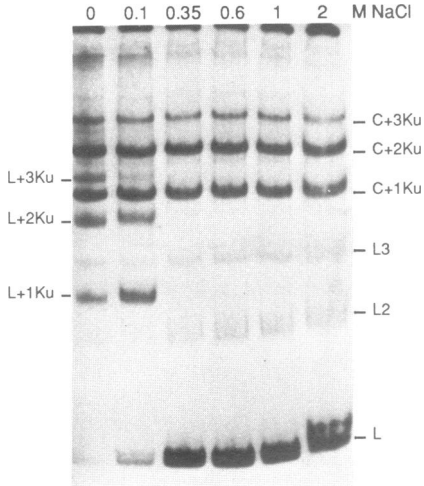

Figure 4. Redistribution of Ku protein as a function of salt concentration. Complexes formed by Ku protein with a labeled 224 bp DNA fragment were incubated with DNA ligase as in Figure 3. To each sample was added a large excess (300 ng) of competitor DNA, plus NaCl to the indicated final concentrations, and incubation was continued. After 30 mn, the samples were diluted if necessary, to lower the NaCl concentration to 100 mM, and analyzed by gel retardation. L, L2, L3: positions of the free monomer, dimer, and trimer of the labeled DNA fragment; L1+1Ku, L1+2Ku, L1+3Ku: positions of the complexes of the linear DNA monomer with 1, 2, or 3 molecules of Ku protein; C+1Ku, C+2Ku, C+3Ku: positions of the complexes of the monomeric DNA circles with 1, 2, or 3 molecules of Ku protein, respectively.

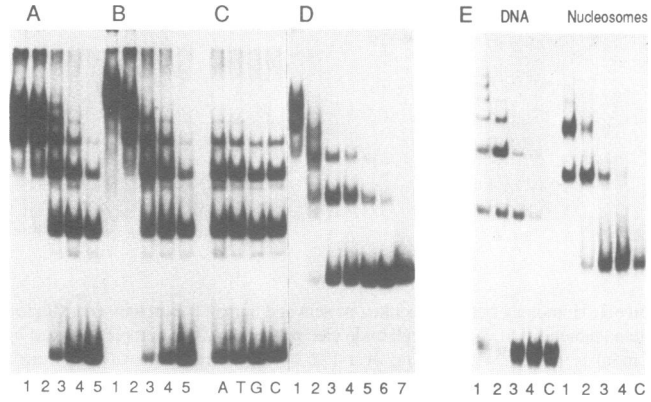

Figure 5. Interactions of Ku protein with different DNA sequences and structures and with nucleosomes. In (A), (B), and (C), complexes of Ku protein with a labeled 224 bp DNA fragment were formed in the presence of different competitor DNA: (A) native, sonicated E. coli DNA: 2, 4, 8, 15, 30 ng in lanes 1−5, respectively; (B) same amounts of the same DNA as in (A), heat-denatured; (C) 15 ng of native E. coli DNA plus 500 ng per sample of the synthetic homopolymers poly dA, poly dT, poly dG et poly dC. In (D) the labeled DNA substrate was a linear, double-stranded, 357 bp-long DNA molecule, terminated at both ends by a hairpin loop. Competitor was E. coli sonicated DNA: 2, 4, 8, 15, 30, 60, 125 ng in lanes 1−7, respectively. In (E), labeled mononucleosomes depleted of histone H1 were used as substrate for interaction with Ku protein, in parallel with labeled DNA extracted from the same mononucleosome preparation. Competitor DNA amounts: 1, 4, 15, 60 ng in lanes 1−4, respectively; lanes C: control, no protein added.

we have also observed some competition by RNA *in vitro* (tRNA, globin mRNA, and total RNA from HeLa cells; data not shown).

Ku protein slides along DNA with no sequence preference

Another question regarding Ku protein is whether it has a preference for remaining at the ends of DNA fragments, or whether it can move freely along DNA, and in this case whether it has preferential binding sites inside the DNA molecules. On one hand, the statistical treatment of electron microscopy data mentioned in ref. 9 suggests that isolated protein molecules can slide along DNA without binding to specific sites. On the other hand, some footprinting experiments have suggested that Ku protein binds to specific sites in promoters (10–12). In Figure 6, Ku protein was first bound to a fragment from plasmid pBR322 that was uniformly labeled by nick-translation and that contained two *Sau*96I sites. After incubation, the complexes were digested with *Sau*96I, yielding a central fragment of 352 bp and two end fragments of 172 and 127 bp. The complexes with these three fragments were analyzed by two dimensional electrophoresis, the first dimension on a retardation gel and the second dimension in the presence of SDS. It is observed (Fig. 6A) that the central 352 bp fragment is complexed with Ku protein, and the proportion of complex relative to free fragment does not vary much from one of the fragments to another. This result is not due to protein redistribution, since the protein does not redistribute under the conditions used (9), and since a large excess of competitor DNA was added before digestion with the restriction enzyme. Therefore Ku protein does not stay at the ends of the fragments but is able to slide apparently freely from the ends to the central part of the DNA molecule.

As this experiment did not reveal any preferential site on a fragment from pBR322, we performed a similar experiment with a DNA sequence that is known to play an important regulatory role in the replication and the transcription of an eucaryotic genome: the control region of SV40. A 557 bp fragment containing the whole control region and the 200 bp upstream part of the late genes was used for the experiment shown in Figure 6B. Cutting this fragment with *Hpa*II releases a 346 bp fragment that contains the control region and a 211 bp fragment that contains sequences from the late genes. It is observed that Ku protein has no preference for any of these two fragments but is equally distributed between the regulatory and the intragenic sequences.

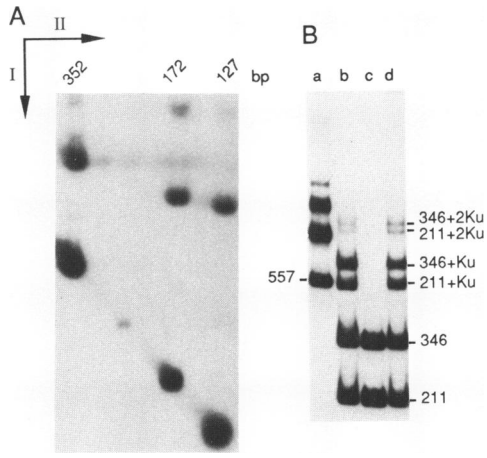

Figure 6. Sliding of Ku protein along DNA fragments. (A) A 651 bp DNA fragment was uniformly labeled by nick-translation, complexed with Ku protein, and cut with *Sau*96I, releasing a central fragment of 352 bp and two end fragments of 172 and 127 bp. The complexes between Ku protein and the 3 fragments were then analyzed by two dimensional electrophoresis: first dimension from top to bottom on a retardation gel, second dimension from left to right on an SDS-4% polyacrylamide gel. (B) a 557 bp DNA fragment from the SV40 genome, containing the control region plus about 200 bp of the late genes, was end-labeled, complexed with Ku protein, and cut with *Hpa*II yielding two fragments of 346 bp (control region) and 211 bp (late genes). Lane a: complexes formed with the 557 bp fragment; lane b: Ku protein was complexed to the 557 bp fragment, then digested with *Hpa*II; lane c: no Ku protein added: free 557 bp fragment digested with *Hpa*II; lane d: complexes formed between Ku protein and the *Hpa*II digest of the 557 bp fragment.

DISCUSSION

We may now draw a detailed picture of the mechanism of interaction of Ku protein with DNA. Free Ku protein can bind DNA only at its ends, but, as we have shown, the structure of the end is relatively unimportant and binding does not involve opening of the double helix. Once bound, Ku protein slides along the DNA leaving the ends free to bind additional protein molecules. The bound protein shows no tendancy to remain at or near the end of a linear DNA fragment but moves freely to occupy, as we have shown, apparently random positions. Bound protein cannot dissociate readily from DNA and does not jump from one fragment to another. Since complexes formed with linear DNA dissociate at much lower salt concentrations than those which involve circular DNA molecules, DNA ends appear to become important again in dissociating the protein from DNA, as we have shown. Although we have no direct evidence that Ku protein surrounds DNA, these properties clearly lead one to think of the protein as a bead threaded on a DNA string.

Given this mechanism of interaction *in vitro*, it is interesting to ask how Ku protein might actually interact with DNA inside the cell. Ku protein was first proposed to play a role in repair of double strand breaks in DNA or in transposition (6,9), and although these possibilities cannot be ruled out, accumulating data suggest that they are unlikely. For example, no indication has been found here or elsewhere that Ku protein can hold two DNA fragments together. It is furthermore improbable that Ku protein could slide for long distances through histones and other proteins, since it has been shown that binding of a second protein along the DNA *in vitro* can interfere with Ku protein binding (9). If Ku protein does play its major role in repair or transposition, therefore, a substantial proportion af this abundant protein would appear to remain unbound in the nucleus at any given time.

Experimental evidence, however, indicates strongly that a large proportion of the protein is bound to DNA at least in interphase nuclei. First, in nuclear extracts, Ku protein is found associated with DNA, not RNA (5,7). Second, Ku protein has been found to be underrepresented on mononucleosomes, as is histone H1, suggesting a possible association of Ku protein with linker DNA (5). This is not likely to be the result of protein redistribution during extraction, since we find that Ku protein binds perfectely well to mononucleosomes *in vitro* . Third, on extraction of nuclei with 2 M NaCl, a large proportion of Ku protein remains insoluble (ref. 16; and data not shown). Finally, immunofluorescence studies show that Ku protein is localized in the nucleus and that its distribution is grossly altered by DNase I, but not by RNase treatment (3,4). Thus, it seems likely that at least a large proportion of Ku protein is bound to DNA during interphase. On the other hand, anti-Ku antibodies do not stain metaphase chromosomes (5). If the protein is more or less

uniformly distributed along DNA during interphase and released at metaphase, it could well be assigned a role in chromosome structure or condensation, in replication, or even in transcription.

Possible regulatory roles for Ku protein have been investigated in several laboratories. Footprints of Ku protein have been observed on the promotors of U1 RNA and transferin receptor genes (10,11). In addition, protein Ku-2 gives footprints on the octamer motif (12). We were unable to detect DNase I footprints on the control region of SV40 at protein/DNA ratios close to or slightly above 1. We also detected no preferential binding sites when Ku protein is allowed to slide along this region, which contains an octamer motif inside the transcription enhancer. Similarly, no preferential binding sites were detected on the adenovirus genome (9). If Ku protein plays a role in the control of gene expression by binding to specific regulatory sequences, this role should not be expected to be limited to just a few sites, especially given the abundance of the protein. We propose an alternative explanation to the footprints, taking into account that they have been observed under very particular conditions using protein/DNA ratios well above unity. These footprints might be the consequence of a modification of DNA topology induced by Ku protein binding (10), as also observed in our ligation experiment in the presence of protein. DNA was observed to be rigidified at high protein/DNA ratio, and even with no binding of the protein at specific sites, such changes in DNA flexibility might modify the cutting pattern of DNase I. It is also possible that the changes in DNA conformation induced by Ku protein influence transcription as observed (11). The large number of proteins described in the literature which give footprints on the octamer motif suggest that this is a DNA element which easely changes conformation as a function of its protein environment, and such changes might, in fact, be an important part of its regulatory role.

The striking property of Ku protein to slide along DNA and to not dissociate until it finds a DNA end leads us to think finally of a possible role for Ku protein in DNA replication. On the condition that binding of Ku protein to DNA is found to end during S phase, one might propose that, during replication, Ku protein bound to DNA is swept away by moving replication forks. It therefore would be absent from newly replicated regions of DNA and might play a role in distinguishing these regions from those yet to be replicated.

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