Binding of Ku Protein to DNA

MEASUREMENT OF AFFINITY FOR ENDS AND DEMONSTRATION OF BINDING TO NICKS

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Ku, also known as nuclear Factor IV, is an abundant nuclear DNA-binding protein which requires free DNA ends for the initial interaction with double-stranded DNA (dsDNA) and can bind at multiple sites along dsDNA in an energy-independent manner. Its function in vivo is unknown, but it has been implicated in both DNA replication and repair and in transcriptional control. We have used an electrophoretic mobility shift assay to further define the DNA binding properties of the Ku protein. Titration of Ku to a fixed amount of any of several target linear dsDNA fragments produced ladders of shifted bands proportional to the length of DNA, confirming the multiple binding activity of Ku and demonstrating its sequence-independent nature. Using a short DNA fragment with one Ku binding site, the binding constant of Ku for dsDNA ends was calculated to be \(2.4 \times 10^9\) M\(^{-1}\). Competitive inhibition experiments confirmed the requirement of a free DNA end for binding by Ku and demonstrated that Ku binds isolated nicks in dsDNA. Nick binding was also observed directly using radiolabeled singly nicked circular DNA. The relative affinities of Ku for specific nick sites and free DNA ends were approximately equal, and nick binding was sequence-independent. Finally, in a study of a possible role for Ku in protecting or repairing damaged DNA, Ku was shown to inhibit the ability of T4 DNA ligase to circularize linear dsDNA molecules, demonstrating that some Ku molecules remain at the DNA terminus rather than translocate. A similar inhibition was not observed at nicks. These experiments document a new DNA binding specificity for Ku and further suggest that the high affinity end and nick binding activity is biologically relevant to its functions in vivo.

The Ku autoantigen is an abundant nuclear DNA-binding protein comprised of 72- and 84-kDa subunits (referred to as p70 and p80) (Mimori et al., 1981, 1986; Reeves, 1985; Yaneva et al., 1986). Its function in vivo is unknown, but in vitro it binds ends of double-stranded DNA without sequence specificity or regard to overhang (Mimori and Hardin, 1986). Native Ku protein contains p70 and p80 subunits in equimolar ratio (Mimori et al., 1986), and Ku subunits synthesized in vitro from cDNA clones assemble in equimolar ratio into complexes which have DNA end binding specificity (Griffith et al., 1992a). Evidence from electron microscopic studies suggests that Ku is a heterodimer (molecular weight 160,000) both in solution and when bound to DNA (de Vries et al., 1989). Ku is identical to nuclear Factor IV, or NFIV, a nuclear DNA end-binding protein which appears to translocate along dsDNA molecules in an energy- and sequence-independent manner (de Vries et al., 1989; Stuver et al., 1990). Ku shows cell-cycle-related movement into and out of the nucleolus (Yaneva and Jhiang, 1991), is found preferentially in "active" chromatin (Yaneva and Busch, 1986), and is a major target of phosphorylation by a newly described DNA-dependent protein kinase (Lees-Miller et al., 1990).

These properties, as well as its abundance, lack of sequence specificity, and affinity for DNA ends, have suggested a role for Ku in basic DNA metabolic processes such as replication, repair, or recombination (de Vries et al., 1989; Mimori and Hardin, 1986; Mimori et al., 1986). However, Ku or Ku-like proteins have been identified in several preparations of transcription factors. These systems include transcriptional activators for the transferrin receptor (Roberts et al., 1989), U1 RNA (Knuth et al., 1990), and an octamer consensus sequence-binding protein (May et al., 1991). Those observations suggest that Ku may have a role in transcription.

We have sought to understand in more detail the DNA binding properties of Ku in an effort to differentiate between these two broad functional categories. Our system uses a competitive electrophoretic mobility shift assay to study the affinity of Ku for various DNA substrates. In these experiments, we have obtained further evidence that Ku binds multiple sites on DNA after binding to ends, in an energy- and sequence-independent manner. Additionally, we have established that Ku has a high affinity for dsDNA ends, that it binds isolated nicks in dsDNA with comparable affinity, and that it can protect DNA from the activity of some DNA-modifying enzymes.

MATERIALS AND METHODS

Plasmid and DNA Fragment Preparations—The plasmid vector pGEM7Zf(+)(3000 bp; Promega, Madison, WI) was grown in JM109 and prepared by CsCl/ethidium bromide centrifugation. Restriction fragments were prepared by digestion of pGEM 7Zf(+) with the appropriate restriction enzymes (Boehringer Mannheim or New England Biolabs) under standard conditions. Specific fragments were excised from acrylamide gels and purified by the "crush-soak" method (Maniatis et al., 1982). Blunt-end linear plasmid used in the ligation inhibition experiment was generated by digestion with EcoRI and filling in with the Klenow fragment of DNA polymerase I and dNTPs.

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1The abbreviations used are: NFIV, nuclear factor IV; bp, base pairs; ds, double-stranded; ss, single-stranded; EthBr, ethidium bromide.
The double-stranded oligonucleotide YP-31 is derived from the Drosophila yolk protein promoter region (Mitsis and Wensink, 1989), with the 5' and 3' ends modified to create BamHI and EcoRI sites for cloning; the sequence of the upper strand is therefore 5'-AATT- CATTGAGGCGCGTGACCTGCAGTGGCTCTCCG-3'. The two strands were synthesized separately by the Yale Pathology Department core facility, and the ds hybrid was formed by boiling and slow cooling to room temperature. DNAs were 5' end-labeled with [γ-32P]ATP (Ameraharn Corp.) and polynucleotide kinase (Boehringer Mannheim). Plasmid-derived DNAs were first dephosphorylated with calf intestine alkaline phosphatase (Boehringer Mannheim); for nicked plasmid molecules or blunt-end restriction fragments, this was performed by Sephadex G-50 (Pharmacia LKB Biotechnology Inc.) spin column chromatography.

Nicked plasmid DNA was generated by digestion with EcoRI at 5 units/μg DNA, at room temperature in a standard buffer in the presence of 100 μg/ml ethidium bromide (EthBr) (Parker et al., 1977). The reaction mixture was separated in a 1% agarose gel containing 0.5 μg/ml EthBr, the band containing Form II (nicked) molecules was excised, and DNA was isolated using Geneclean (Bio101, La Jolla, CA). These preparations were routinely >95% Form II as assessed by relative band intensities after electrophoresis through ethidium bromide-containing agarose gels. For the experiment in Fig. 5, AflIII digestion conditions were 56°C for more than 37°C. Unincorporated label was removed by Sephadex G-50 (Pharmacia LKB Biotechnology Inc.) spin column chromatography.

RESULTS

Ku Binds Multiple Sites along Linear dsDNA after Binding to Free Ends.—In order to compare the DNA binding behavior of immunofluorescence-purified Ku to that of biochemically purified NFIV (de Vries et al., 1989), we titrated purified Ku protein with a fixed amount of a radiolabeled linear plasmid restriction fragment in binding buffer. The DNA-protein complexes were separated from unbound DNA in native high ionic strength low percentage polyacrylamide gels. In preliminary experiments we observed that dsDNA molecules were quantitatively bound by Ku protein to form a "ladder" of progressively shifted bands as increasing amounts of Ku were added. This ladder pattern was unchanged in the presence of 10 mM EDTA or ATP at 100 μM or 1 mM, implying that divalent cations or ATP are not required (data not shown). As demonstrated by the examples in Fig. 1, ladders were seen with every plasmid restriction fragment tested, suggesting an absence of sequence specificity in this activity.

The ladder of bands observed in the mobility shift assays could represent multimers of DNA fragments linked by Ku molecules or single DNA fragments bound by increasing numbers of Ku protein molecules. To differentiate between these possibilities, titration assays were performed with DNA fragments of different lengths. As shown in Fig. 1, the maximum number of "rungs" in the banding ladder increased as the DNA probe was lengthened. A single shifted band formed with the 31-bp probe (A), a maximum of five bands developed with a 166-bp probe (B, left), and at least eight bands occurred with a 299-bp probe (B, right). The simplest explanation for this pattern is that the ladder represents DNA-protein complexes of the form DNA • Kuₙ, where n varies from 1 to a maximum which is dependent on DNA length. These results

From the equation, 
\[
K_{\text{wu}} = \frac{([\text{DNA}:\text{Ku}])}{([\text{DNA}]_{\text{tot}})[\text{Ku}]_{\text{tot}})},
\]

at 50% DNA bound
\[
([\text{DNA}:\text{Ku}]) = ([\text{DNA}]_{\text{tot}}) - ([\text{DNA}]_{\text{free}})
\]

and

\[
K_{\text{wu}} = 1 - \frac{1}{2}([\text{DNA}]_{\text{free}})
\]

Provided protein is in excess, the value of [Ku]ₙ can be calculated from the input Ku and DNA concentrations at 50% DNA bound using the following relationship.

\[
([\text{Ku}]_{\text{wu}}) = ([\text{Ku}]_{\text{tot}}) - ([\text{DNA}])
\]

\[
=(([\text{Ku}]_{\text{tot}}) - \frac{1}{2}([\text{DNA}]_{\text{free}}))
\]
Fig. 1. Titration of Ku on linear DNA fragments of varying lengths. Serial dilutions of an immunoaffinity-purified Ku protein preparation were incubated with a fixed amount of a radiolabeled dsDNA probe as described under "Materials and Methods." After 30 min at room temperature, aliquots of each sample were analyzed by electrophoresis through high ionic strength nondenaturing polyacrylamide gels and autoradiography. DNA probes used were a synthetic 31-bp ds oligonucleotide named YP-31 (A), a 166-bp DdeI restriction fragment of pGEM7Zf(+) (B, left), and a 299-bp HinfI restriction fragment of the same plasmid (B, right). Amount of Ku used in femtomoles is indicated along the top (corrected for percent functional molecules; see legend to Fig. 2), and mobility of the free probes and of Ku-DNA complexes of increasing sizes is indicated along the sides.

suggest a maximum binding capacity of one Ku molecule/30-35 bp, a figure consistent with previous DNase I footprinting studies (Mimori and Hardin, 1986). These results also confirm those obtained by deVries et al. (1989) in their studies of NFIV. A similar banding ladder was also seen by Roberts et al. (1989) in their studies of transferrin receptor transcription factors 1 and 2.

Since Ku requires a free DNA end for the initial interaction with DNA, banding ladders have been interpreted by others as suggesting the Ku protein translocates along DNA molecules after binding an end (de Vries et al., 1989). Although our results are consistent with this interpretation, they are also consistent with a model in which the initial binding of a Ku molecule at a DNA end enables further Ku molecules to bind internal DNA sites.

A doublet is usually seen in the banding ladder at the position corresponding to n = 1 (Fig. 1). Doublets are not resolved in higher order complexes and are seen with any of several different DNA fragments used as probe. Doublets were also seen in our earlier studies of the interaction of radiolabeled, in vitro synthesized Ku protein with DNA (Griffith et al., 1992a). They may represent differential mobility of complexes with a single Ku molecule end-bound or internally situated after translocation or binding by two different forms of Ku (e.g. differing in phosphorylation state). Since the doublet is observed even with the 31-bp probe which appears to contain only a single Ku binding site (Fig. 1A), the latter interpretation may be more likely.

Ku Has a High Affinity for Free dsDNA Ends—Since the 31-bp oligonucleotide, YP-31, forms only a single complex with Ku, it can be used in a band shift assay to determine an affinity constant for the initial interaction of Ku with DNA (Fried and Crothers, 1981). A fixed amount of purified Ku was incubated with increasing amounts of cold YP-31 in the presence of a small amount of radiolabeled YP-31 tracer. After electrophoresis and autoradiography, the proportion of DNA bound was quantified using laser densitometry and plotted as a function of total DNA concentration (Fig. 2). The 50% binding point was used to determine an affinity constant as described under "Materials and Methods." Using this approach, the binding constant of Ku for YP-31 is $2.4 \times 10^9$ M$^{-1}$. This binding constant is reasonably large; it is intermediate between that measured by band shift assay for two sequence-specific DNA binding proteins, Escherichia coli cAMP-binding protein (CAP) and the lac repressor (Fried and Crothers, 1981, 1984), and that determined by time-resolved fluorescence spectroscopy for the Klenow fragment of DNA polymerase I for a nonspecific oligonucleotide (Guest et al., 1991).

We were concerned that the minor doublet band observed in binding of Ku to YP-31 might affect the calculation of $K_m$ and the relative affinities determined in the next section.
whether the observed binding of Ku to single-strand nicks in dsDNA is of sufficient affinity to suggest a physiological role. Therefore, we undertook a quantitative comparison of the ability of nicked, linear, and closed circular plasmid DNA to inhibit binding of Ku to oligonucleotide YP-31. Band intensities were quantified with laser densitometry and percent residual binding plotted as a function of inhibitor concentration. The results are shown in Fig. 4. The 50% inhibitory concentration for nicked DNA is approximately 3.6-fold higher than that for linear DNA (Fig. 4, open boxes versus closed triangles), but this difference is reduced to 1.8-fold when correction is made for the number of presumed access sites (two ends per molecule for linear DNA versus one strand break per molecule for nicked DNA). Therefore, within the limits of this assay, Ku has nearly equivalent affinity for free dsDNA ends and single-strand nick sites in dsDNA.

Ku Binds Nicks Generated by Several Restriction Enzymes—In order to rule out the possibility that the observed nick binding activity was unique to the DNA sequence around the EcoRI site in the pGEM plasmid, we sought to demonstrate that Ku could bind nicks generated by other restriction enzymes as well. Conditions were found under which several other restriction enzymes could also generate nicked molecules from supercoiled pGEM 7ZI(+). Nicking digestions were performed with EcoRI (1 site), AflIII (2 sites), and MspI (14 sites), and Form II molecules gel-purified as described under “Materials and Methods.” Agarose gel electrophoresis and ethidium bromide staining demonstrated that all Form II preparations were >95% pure (data not shown). These preparations were then used in competitive inhibition mobility shift assays as shown in Fig. 5. Plasmids nicked by any of the three enzymes were able to compete for binding by Ku (lanes 5–16). Since the recognition sites of each enzyme are found in different positions around the plasmid, this observation confirms that Ku binds to nicks in DNA with no sequence specificity.

Direct Binding by Ku to Nicked Circular dsDNA Molecules Can Be Demonstrated—We wished to directly demonstrate binding by Ku to nicked DNA molecules in addition to the indirect evidence provided by competitive inhibition. Therefore, preparations of pGEM 7ZI(+) linearized or nicked at the EcoRI site were radiolabeled using [γ-32P]ATP and T4 polynucleotide kinase and used directly in mobility shift assays (Fig. 6). Although the resolution of a ladder of bands is not sharp with these 3000-bp DNA molecules, an alteration in mobility of the radiolabeled DNA can clearly be observed with either linear (Fig. 6A) or nicked (Fig. 6B) plasmid DNA as increasing amounts of Ku protein are added. The absence of a series of shifted bands precludes quantitation of the Ku-DNA complexes possible with shorter DNA fragments (Figs. 1–4). However, in both cases the observed mobility alteration can be nearly completely inhibited by addition of a 50-fold excess of cold competitor DNA (lanes 8 and 9 in both panels). We conclude that the shift is due to specific binding by Ku to the nicked or linear plasmid molecules.

Ku Inhibits the Action of T4 DNA Ligase on Linearized Plasmid DNA—In an effort to investigate possible functional correlates of the end and nick binding properties of Ku, we studied the effect of the protein on ligation of DNA molecules in vitro. We used an assay modified from studies by Thode et al. (1990) on nonhomologous recombination in Xenopus. Plasmid DNA, either linear with "sticky" or blunt ends, or nicked, was preincubated with varying amounts of Ku, and T4 DNA ligase was added. Ligation products were analyzed by electrophoresis in EthBr/agarose gels and Southern hybridization. The results are shown in Fig. 7. In the absence of Ku, linear...
of Ku on the ends of the linear DNA and consequent interference with the function of ligase. These findings also suggest that, although the Ku protein may be able to translocate along the DNA molecule after binding to a free end, not all molecules do so, and some remain bound to ends.

Curiously, the same interference with ligation was not observed in the nicked molecules (Fig. 7, lanes 12–14). Even at relatively high protein:DNA ratios, closure of nicks by T4 DNA ligase was unaffected. This finding also rules out the possibility that a nonspecific inhibitor of ligase was present in the Ku preparation which might have inhibited ligation of linear DNA (lanes 2–11).

DISCUSSION

Since the initial description of the Ku autoantigen in 1981 (Mimori et al., 1981), much has been learned about its structure and properties (Allaway et al., 1990; Chan et al., 1989; Griffith et al., 1992a; Mimori and Hardin, 1986; Mimori et al., 1986; Reeves and Sthoeger, 1989; Yaneva et al., 1989). However, definition of its functional role in vivo has remained elusive. We have sought clues to this problem by focusing initially on a detailed characterization of the DNA binding properties of Ku. Our demonstration in mobility shift assays of “banding ladders” which vary in length depending on the length of the probe DNA (Fig. 1) extends our earlier work which established that Ku binds free ends of dsDNA (Mimori and Hardin, 1986) and is consistent with the results of de Vries et al. (1989) which suggested Ku then translocates along a dsDNA molecule in an energy- and sequence-independent manner. Our results indicate that approximately 30–35 bp are required per Ku molecule, consistent with our earlier footprinting studies (Mimori and Hardin, 1986) and with chemical DNA footprinting and transmission electron microscopy performed by de Vries et al. (1989). Similar banding ladders are seen in our assay regardless of the DNA fragment tested, emphasizing the sequence-independent nature of this activity.

Bandaging ladders would also be seen if binding of an initial Ku molecule to DNA enhanced the ability of subsequent molecules to bind to internal sites, for instance, due to cooperativity of binding or by provision of a nucleation site. Our mobility shift experiments do not rule out this possibility. However, in their studies of NFIV, de Vries et al. (1989) found that the second step of binding to DNA was rate-limiting and more strongly temperature-dependent than the first step (end-binding); furthermore, by electron microscopy, Ku molecules were found widely spaced at irregular intervals on DNA at low protein:DNA ratios. Both these findings seem more
AflIII with radiolabeled 166-bp competitors and DNA. Plasmid DNA radiolabeled, and used as substrate for binding with various concentrations of Ku protein. Assay and electrophoresis conditions were largely as described in the legend to Fig. 1A, except that approximately 0.4 fmol of linear and 0.6 fmol of nicked DNA were used in each binding reaction. A shows the titration against end-labeled linear plasmid DNA (lin) and B that against nick-labeled open circular (oc) DNA. In the competitive inhibition lanes (lanes 8 and 9 of each panel), 25 fmol of unlabeled linear plasmid or open circular DNA was present at the start of incubation.

The method used to determine the percent active protein (Fried and Crothers, 1981) requires knowledge of the subunit structure of the protein when in solution and when bound to DNA. We have assumed that Ku in both states is a heterodimer comprised of one of each subunit (total molecular weight 156,000), based principally on the electron microscopic evidence of de Vries et al. (1989), who demonstrated protein particles of a calculated volume corresponding to a protein molecular weight of 160,000 whether free or bound to DNA.

Several arguments indicate that one Ku molecule occupies about 30 bp of DNA and suggest that the shifted band observed with the YP-31 probe (and the first shifted band with longer probes) represents DNA complexed with one and not two Ku molecules. First, the probe is similar in size to the DNA segment protected by Ku in D NAse I or chemical footprinting (Mimori et al., 1986; de Vries et al., 1989), and to the spacing of Ku molecules on DNA seen in transmission electron microscopy (de Vries et al., 1989). Second, titrations of Ku on YP-31 never demonstrate a shifted band of intermediate mobility (Fig. 1A), which might be expected if two Ku molecules could bind the fragment. Third, the banding ladders with longer probes provide a better fit to a semi-log plot of molecular weight to relative mobility if the first shifted band is presumed to contain one Ku molecule (not shown). Fourth, when YP-31 is labeled with biotin at the terminal EcoRI site leaving the BamHI terminal free, only one Ku-DNA complex is formed whether or not the EcoRI end is blocked with streptavidin (data not shown). Therefore, we believe that the smallest shifted complexes represent one Ku molecule per DNA fragment.

The use of the band shift assay to determine binding constants for DNA-protein interactions is well grounded theoretically as well as experimentally (e.g. Ceglarek and Revzin, 1989). Ku demonstrates two types of interaction with DNA: initial binding to an end or nick and subsequent internal binding (Figs. 1 and 6; de Vries et al., 1989). Our use of a short DNA probe was intended to permit us to focus on a quantitative analysis of the first interaction. Further studies may permit a more detailed analysis of the second interaction, including determination of possible cooperativity in binding of additional Ku molecules to longer DNA substrates.

By competitive inhibition and direct binding, we have demonstrated that Ku can also recognize isolated nicks in dsDNA molecules (Figs. 3 and 6). These nicks are simple single strand

![Graph](image-url)

**Fig. 5.** Competitive inhibition of binding by Ku to dsDNA with singly and multiply nicked circular dsDNA. Ku was incubated with radiolabeled 166-bp DdeI restriction fragment in the presence of increasing amounts of pGEM7Zf(+) nicked with EcoRI (lanes 5–8), AflIII (lanes 9–12), or MspI (lanes 13–16) or undigested (lanes 1–4). Lane 17 demonstrates migration of probe with Ku in the absence of competitors and lane 18 the migration of probe alone. Amount in nanograms of each competitor is indicated along the top.

**Fig. 6.** Titration of Ku on linearized and nicked plasmid DNA. pGEM7Zf(+) DNA was linearized or nicked at the EcoRI site, radiolabeled, and used as substrate for binding with various concentrations of Ku protein. By mobility shift assay using a ds oligonucleotide containing one binding site, we have shown that Ku recognizes DNA ends with high affinity, $K_{eq} = 2.4 \times 10^8$ M$^{-1}$ (Fig. 2). These data were obtained at room temperature and 150 mm NaCl; possible salt or temperature dependence were not investigated. With cumulative uncertainties because of limitations in the quantitation of protein and DNA concentrations, we believe this estimate to be accurate to within a factor of two. Even with this relatively high binding constant, this binding constant is consistent with values obtained for other DNA-binding proteins. It is about 20-fold greater than the $K_d$ of Klenow fragment for a DNA oligonucleotide, determined by time-resolved fluorescence spectroscopy to be 7.9 nM (Guest et al., 1991) and about 20-fold less than the binding constants determined by band shift assay for two sequence-specific DNA binding proteins: E. coli cAMP-binding protein (CAP) (8.4 $\times$ 10$^{10}$ M$^{-1}$) (Fried and Crothers, 1984) and the binding of lac repressor to the so-called first operator site (O1) of the lac operon (5.7 $\times$ 10$^{10}$ M$^{-1}$) (Fried and Crothers, 1981).

The smallest shifted complexes represent one Ku molecule per DNA fragment.

By competitive inhibition and direct binding, we have demonstrated that Ku can also recognize isolated nicks in dsDNA molecules (Figs. 3 and 6). These nicks are simple single strand
DNA End and Nick Binding by Ku

| DNA End         | linear EcoRI-end | linear blunt end | nicked |
|-----------------|------------------|------------------|--------|
| Ku              | 1  0.5  0.25  0  | 0  0  0  0  | 1  0.5  0.25  0  |
| ligase          | +    +    +    + | -    +    +    + | +    +    +    + |
| oc              | +    +    +    + | -    +    +    + | +    +    +    + |
| lin             | +    +    +    + | -    +    +    + | +    +    +    + |
| ccc             | +    +    +    + | -    +    +    + | +    +    +    + |

Fig. 7. Effect of Ku on ligation of linear and nicked plasmid DNA by T4 DNA ligase. Three DNA substrates (linear, EcoRI end; linear, blunt end; and nicked) were preincubated with or without Ku. A portion of each reaction was then treated with or without T4 DNA ligase. DNA products were analyzed by electrophoresis through 1% agarose gels containing 0.5 μg/ml ethidium bromide and Southern hybridization using as probe-radiolabeled pGEM7Zf(+) DNA. Lane 1 is unmodified plasmid. Mobility in this gel system of nicked (open circular, oc), linear (lin), and covalently closed circular (ccc) DNA is indicated on the left. The amount of Ku used in each preincubation is shown along the top in picomoles (corrected for percent functional molecules), as is the presence (+) or absence (−) of ligase in the second step.

breaks in a phosphodiester bond with no loss of nucleotides (and hence are not gaps), since the molecules can be reclosed with T4 DNA ligase (Fig. 7). Although the nick binding activity was previously alluded to in discussion (May et al., 1991), it has been characterized in detail here for the first time. The affinity for isolated nicks is roughly equal to that for free ends (Fig. 4) and is independent of the surrounding sequence, since circular dsDNA nicked by different restriction enzymes at various defined sites can all bind Ku (Fig. 5).

On the other hand, the binding seen to M13 ssDNA circles (Fig. 3D) and ssDNA oligonucleotides (not shown) is probably of much lower affinity. However, we do not know what specific structure is recognized by Ku in single-stranded M13 DNA. Competition by M13 was seen in previous work (Mimori and Hardin, 1986) using a nitrocellulose filter binding assay. Since this binding occurs in the absence of a free end, the titration curve must represent competition by internal sites. Its apparent effectiveness may be due to the large molar excess of low affinity binding sites the large ssDNA circle represents. In other experiments, we have observed only minimal binding of Ku to an end-labeled 31-base ss oligonucleotide (one of the strands of YP-21), and competitive inhibition of binding to YP-31 required a several hundredfold excess of the ss oligonucleotide (data not shown).

We do not know if the binding to nicks and ends occurs by identical mechanisms. Although the affinities and sequence independence are similar, we observed a possible difference in the effect of Ku on re-ligation of the two structures (Fig. 7). It remains to be determined whether the dissociation kinetics of Ku at ends and nicks differ or if the Ku-DNA complexes themselves are different. However, it is possible that the lack of inhibition of nick closure simply results from the more favorable kinetics of this essentially unimolecular reaction compared with the bimolecular kinetics of end ligation and that the conditions employed were insufficient to demonstrate an effect at nicks. Experiments are underway to resolve these questions.

Because of the similarities in the nick and end binding activities, we propose that part of Ku's function in vivo involves recognition of both structures. The ability to specifically recognize nicks is seen in ligases, polymerases, topoisomerases, and other DNA-modifying enzymes lacking sequence specificity but has not been shown to be part of the DNA binding properties of well characterized sequence-specific proteins (Johnson and McKnight, 1989). Nick and double-strand DNA breaks in chromatin are found in vivo, particularly in cells undergoing cell division and DNA replication (Iseki, 1986; Taki et al., 1990). One possible function for Ku may be to protect these sites from undesired modification or nonhomologous recombination or to assist repair enzymes in correctly assembling the chromosomal structure. Its abundance (0.5–1.0 × 10⁶ molecules/cell (Mimori et al., 1986)), ubiquity, high affinity, and the lack of sequence specificity in its DNA binding properties are consistent with such a function.

This leaves open the relationship between these properties of Ku and the apparent specificity for sequences implicated in transcriptional activation described by other groups (Knuth et al., 1990; May et al., 1991; Roberts et al., 1989). In these systems, Ku or a protein much like Ku in amino acid sequence was isolated during attempts to purify proteins binding to the transferrin receptor promoter, the proximal sequence element (PSE1) in U1 RNA transcription, and an octamer consensus sequence. Although it is possible the Ku protein itself has sequence-specific DNA binding properties, we favor the hypothesis that Ku may provide a "carrier" or nonspecific DNA binding activity to which other subunits provide specificity or serve a bridging function between specific factors and other transcriptional or regulatory proteins. Such a bridging activity has recently been described between TFIIID and RNA polymerase II (Flanagan et al., 1991). The combination of nick or end recognition and translocation also suggest a scanning or processivity function, similar to that of the β subunit of *Escherichia coli* DNA polymerase III, which slides along DNA and serves as an anchor for other subunits of the polymerase (Stukenberg et al., 1991). Our results may suggest that Ku is also a member of this class of proteins.

Finally, the Ku protein may function both as a protein involved in DNA metabolism and in transcription, perhaps varying with cell cycle and its phosphorylation state, which have been shown to be related (Stuiver et al., 1991). If so, Ku would join the ranks of a small but growing number of nuclear proteins (e.g. NF-III/Oct-1 and NF-I/CTF) with roles in both transcription and DNA replication (Herendeen et al., 1989; Mohr et al., 1990; O'Neill et al., 1988; Santoro et al., 1988).

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