Ku Selectively Transfers between DNA Molecules with Homologous Ends*

(Received for publication, August 5, 1996, and in revised form, November 18, 1996)

Tonya M. Bliss‡ and David P. Lane§

From the Cancer Research Campaign Cell Transformation Research Group, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, United Kingdom

Double strand break repair and V(D)J recombination in mammalian cells require the function of the Ku protein complex and the DNA-dependent protein kinase. The DNA-dependent protein kinase is targeted to DNA through its interaction with the Ku protein complex, and thus the specificity of template recognition in the repair and recombination reactions depend on Ku. We have studied Ku binding to DNA using competitive gel shift analysis. We find that Ku bound to one DNA molecule can transfer directly to another DNA molecule when the two DNA molecules have homologous ends containing a minimum of four matched bases. This remarkable reaction can give a false impression of sequence specificity of Ku DNA binding under certain assay conditions. A model is proposed for the DNA binding function of Ku on the basis of these results and the discovery of a novel type of DNA-Ku complex formed at high Ku/DNA ratios is discussed.

The Ku autoantigen is a nuclear DNA-binding protein composed of two subunits, one of approximately 70 kDa and the other 80 kDa (Ku70 and Ku80, respectively) (1, 2). Once bound to DNA, Ku can interact with the catalytic subunit of the DNA-dependent protein kinase (DNA-PK), and together they constitute the active kinase (3–5). Ku is an abundant protein (estimated 4 × 10¹⁰ copies/HeLa nucleus; Ref. 2) found in most human cells studied so far (6, 7). Homologues of Ku have also been reported in monkeys (8), Xenopus (9), yeast (10), Drosophila (11), and rodents (12, 13), although in rodents at least, the concentration of Ku protein is not as high as in humans (13).

Initial studies into the mechanism of interaction of Ku with DNA showed that Ku binds to the ends of double-stranded DNA fragments without any preference for the nature of the ends; Ku does not seem to discriminate between blunt ends and ends with either a 5′- or 3′-overhang (14–18). With longer DNA fragments, several molecules of Ku can bind to the DNA to form a regular DNA-multimeric protein complex that appears as a “beads on a string” structure by electron microscopy (15) or as a ladder of bands in a gel shift assay (8, 15, 17). As well as binding DNA ends, Ku can also bind to regions of DNA containing a double strand to single strand transition (8, 18). Such a DNA secondary structure is postulated to be found in DNA intermediates formed during recombination (19, 20). The presence of secondary structure would explain why the M13 and poly(dI-dC) single-stranded DNA could bind Ku, whereas other single-stranded DNA molecules could not (8, 14).

There is debate in the literature about whether Ku can also bind DNA in a sequence-dependent manner. Several groups have reported that Ku interacts with specific sites in several gene promoters to play a role in the transcription of these genes. A competitive DNA binding assay revealed that Ku70 bound specifically to the E3 motif of the T cell receptor β-chain gene (21) and footprinting studies showed that Ku-2, a protein similar but not identical to Ku, bound to the eukaryotic octamer motif (22). Protein preparations termed TREF and proximal sequence element-binding protein 1 (PSE1), which are similar if not identical to Ku, appear to interact specifically with the transferrin receptor transcriptional control element (TRE) in the promoter of the human transferrin gene (23) and the proximal sequence element of the U1 small RNA gene (24), respectively. Ku has also been reported to bind to the promoter region of rDNA and to influence transcription from this promoter (25, 26). Recently Ku was also shown to bind the negative regulatory element sequence (NRE1) in the long terminal repeat of the mouse mammary tumor virus and, through the actions of DNA-PK, to repress viral transcription (27).

The DNA binding characteristics of Ku suggested a role for the protein in recombination and DNA repair. These speculations were recently confirmed by work with hamster cell lines that were hypersensitive to agents that cause double strand breaks, their phenotype being, at least partially, due to an inability to repair the DNA breaks. These mutant cell lines fall into four distinct ionizing radiation (IR) complementation groups (IR4–7). Introduction of Ku80 cDNA corrected for both the radiosensitivity and V(D)J recombination defects in xrs-6 cells, which are in ionizing radiation complementation group 5 (IR5) (28–30), indicating that Ku80 is the defective gene in IR5. Furthermore, the gene for the catalytic subunit of DNA-PK corrected for the same defects in cells derived from the severe combined immune deficiency (SCID) mouse and V3 cells, which are grouped in IR7 (5, 31–33). These results suggest that Ku and DNA-PK are essential for DNA double strand break repair and V(D)J recombination. Common to both these pathways is the bringing together and joining of DNA ends. Ku and DNA-PK are postulated to be involved in bringing DNA ends together, but their exact mechanism of action is not understood. Clearly, the mechanism of Ku binding to DNA is fundamental to its function.

This study looks at the nature of Ku binding to DNA and shows that it forms two distinguishable interactions with DNA. In addition, we show that Ku can transfer directly from one DNA molecule to another. The implications of our results for

* This work was funded by the Cancer Research Campaign and Howard Hughes Medical Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Wellcome/Cancer Research Campaign Institute, Tennis Court Road, Cambridge CB2 1QR, United Kingdom. Tel.: 44-1223-344103; Fax: 44-1223-334089.

§ Gibb Fellow of the Cancer Research Campaign and a Howard Hughes International Scholar.

The abbreviations used are: DNA-PK, DNA-dependent protein kinase; EMSA, electrophoretic mobility shift assay; dsb, double strand break.

This paper is available on line at http://www-jbc.stanford.edu/jbc/
DNA double strand break repair and V(D)J recombination are discussed. Finally, we also demonstrate that competitive DNA binding assays can give a false impression of Ku binding to DNA in a sequence-specific manner. We propose a set of criteria that should be met when establishing sequence specificity of Ku binding to DNA.

EXPERIMENTAL PROCEDURES

Reagents and Proteins—Bacterially expressed, wild type human p53 was purified as described previously (34). Purified Ku (described below) was biotinylated using the Amersham International plc. ECL protein biotinylation reaction module. Ku (3.5 μg) was incubated for 1 h at room temperature with 3.6 μl of the biotinylation reagent in a total volume of 200 μl.

Purification of Ku—HeLa cells were resuspended in 40 ml of lysis buffer (phosphate-buffered saline containing 1% Nonidet P-40, 5 mM dithiothreitol, 1 mM benzamidine) for every 10 ml of cell pellet. This and all subsequent procedures were carried out at 4 °C. After incubation for 30 min, lysates were centrifuged at 14,000 rpm for 25 min, and the supernatant, containing the soluble Ku, was fractionated using 8% polyethylene glycol precipitation. The resultant precipitate was dissolved in buffer B (25 mM HEPES, pH 8, 15% (v/v) glycerol, 0.1 mM EDTA, 0.1% (v/v) Triton X-100, 5 mM dithiothreitol, 1 mM benzamidine) containing 10 mM KCl to give a 10 mg/ml solution of the protein that was applied to a Q50 anion exchange column (150 μl) equilibrated in buffer B containing 10 mM KCl and eluted with a linear gradient of 10–70 mM KCl in buffer B. Fractions containing Ku, as assessed by an electrophoretic mobility shift assay (described below), were pooled and dialyzed against buffer B containing 50 mM KCl. The dialyzed sample was applied to a heparin- Sepharose column equilibrated in buffer B containing 10 mM KCl and eluted with a linear gradient of 10–700 mM KCl in buffer B. Ku-containing fractions were pooled and dialyzed against buffer B containing 20 mM KCl and applied to a 1-ml DNA affinity column equilibrated in buffer B containing 20 mM KCl. The DNA affinity column was constructed by ligating the PG oligonucleotide with a biotinylated PG oligonucleotide in a molar ratio of 10:1 to give various lengths of PG chains containing the biotin moiety (35). These concatenated PG oligonucleotides were attached to a streptavidin-agarose matrix through the high affinity biotin-streptavidin bond. Approximately 25% of the available sites were bound by DNA. The column was eluted with a linear gradient of 0.05–1 M KCl in buffer B in 20 column volumes. At 0.2 M KCl, the gradient was held for 5 column volumes to wash off proteins bound nonspecifically to the agarose matrix. After the final column of the Ku protein was judged to be at least 95% pure as determined by a Coomassie Blue-stained SDS-polyacrylamide gel.

Oligonucleotides and Preparation of Duplex Substrates—The synthetic, complementary oligonucleotides for the more important duplex DNA substrates are as follows (lower case letters represent non-bonded nucleotides): PG (36), 5′-agtTAGACATCGCTAGACGTACGCTA-3′ and 5′-agtTACGGATCTAGAGCCTAGCTA-3′; PGII (5′-gtgcaAGACGATCGCTAGACGTACGCTA-3′ and 5′-tgtagcGATCGCTAGACGTACGCTA-3′); TL-DNA, 5′-tagttAGAACGGTTGGAACGTCTA-3′; TL3, 5′-agtTACGATACAGCCAGCGGTCTAGCTA-3′; TL4, 5′-agtTGCAGATACAGCCAGCGGTCTAGCTA-3′; and TLnc, 5′-agtTACGATACAGCCAGCGGTCTAGCTA-3′ and 5′-gtgcaAGACGATCGCTAGACGTACGCTA-3′.

Details of other oligonucleotide sequences are available on request. Complementary oligonucleotides were hybridized in 0.1 M HEPES, pH 8 (containing 100 mM MgCl2, 20 mM dithiothreitol), by heating to 80 °C and slow cooling. Complementary single-stranded oligonucleotides were 5′-labeled with 32P-PITP using T4 polynucleotide kinase (34), hybridized as above, phenol extracted, and separated from unincorporated ATP by gel filtration.

Electrophoretic Mobility Shift Assay (EMSA)—DNA binding assays were performed as described previously (34). In general, 20 μl of DNA binding buffer (40 mM HEPES, pH 7.6, containing 20% (v/v) glycerol, 5 mM dithiothreitol, 1 mM bovine serum albumin, 0.1% (v/v) Triton X-100, 50 mM KCl, 0.5 mM EDTA, 10 mM MgCl2) was mixed with the relevant radiolabeled DNA (~2–4 ng) and the indicated amounts of purified Ku protein. For competitive DNA binding assays the competitor DNA was added after the above reaction had been incubated on ice for 2–5 min, except in Fig. 3, for which the competitor DNA was added to the reaction before the Ku protein, as indicated. Incubations followed on ice for 15–30 min. In Fig. 9 there was a 10-min incubation period between the addition of each component of the reaction. After the addition of streptavidin the reaction was incubated for a further 10 min before being loaded onto the gel. Reaction products were loaded onto a 4% polyacrylamide gel containing 0.56 × Tris borate/EDTA (TBE) and 0.1% Triton X-100, which had undergone pre-electrophoresis at 150 V for 5 min at 4 °C in a 0.33 × TBE/0.1% Triton X-100 running buffer. Electrophoresis was continued at 200 V for 90 min at 4 °C. Gels were dried and exposed to x-ray film at −70 °C.

RESULTS

Apparent Sequence Specificity of Ku DNA Binding

Ku is a DNA-binding protein known to interact with the ends of double-stranded DNA fragments, but there is some debate in the literature about whether Ku can also bind DNA in a sequence-dependent manner. Several groups have used competitive DNA binding studies and footprint assays to demonstrate Ku specifically binding to several unrelated DNA sequences (21, 23–25, 27). In a similar manner we found by competitive DNA binding studies that Ku in HeLa cell extracts appeared to bind sequence specifically to the PG DNA sequence, a sequence bound specifically by the tumor suppressor protein p53 (36, 37). To determine whether this specificity was an inherent property of Ku or imposed on Ku by another protein in the cell extract (23, 38), Ku protein was purified to homogeneity from HeLa cells, and its DNA binding properties were assessed in an EMSA (Fig. 1). The linear oligonucleotide PG was used as the labeled DNA with unlabeled PG or an unrelated linear oligonucleotide (TL) used as competitor DNA. The PG DNA competed for both Ku and p53 binding to the labeled probe, whereas the TL oligonucleotide had no effect (Fig. 1). It therefore appeared that Ku itself was binding to DNA in a sequence-specific manner, showing a preference for the PG sequence over the TL sequence. However, when a plasmid containing the PG sequence was added in excess, no competition was observed (data not shown), indicating that Ku did not recognize the PG sequence in the context of a plasmid. Furthermore, linear oligonucleotides containing mutated PG sequences competed with Ku binding to the PG probe, whereas, in contrast, none of the mutated PG sequences competed for p53 binding to PG (data not shown). This result implies that the precise PG sequence is
critical for p53 binding but does not appear to be important for Ku to bind DNA. Taking this result in conjunction with the result showing that Ku does not bind the PG sequence in the context of a plasmid, it suggests that the presence of DNA ends is more important for Ku to bind DNA than the actual internal DNA sequence. However, the presence of DNA ends per se appeared not to be sufficient for Ku to bind DNA, since in Fig. 1 the linear TL oligonucleotide did not compete for Ku binding to the labeled PG sequence despite having free DNA ends.

Apparent Specificity of Ku DNA End Binding Activity

If DNA ends are important for Ku to bind DNA, why does the linear TL oligonucleotide not compete for Ku binding in Fig. 1? Comparing the ends of the various competitor oligonucleotides revealed that the PG and the mutated PG oligonucleotides that compete for Ku binding to the PG DNA have identical end sequences, whereas the TL oligonucleotide that does not compete for Ku binding has a different end structure (Table I). Thus, it was possible that Ku recognized the DNA end sequence and that the difference in the end sequences of the PG and TL oligonucleotides (Table I) could account for the apparent difference in their ability to bind Ku (Fig. 1). To test this, oligonucleotides were synthesized to create a TL sequence with the same ends as the PG probe (to give TL4) and vice versa (to give PG II) (Table I). Fig. 2 shows that in a competitive DNA binding assay, the new TL oligonucleotide (TL4) does compete for Ku binding (Fig. 2A), whereas the new PG DNA (PG II) does not (Fig. 2B). Therefore, changing the ends of the PG and TL oligonucleotides changed their ability to bind Ku. In contrast, the PG sequence but not the TL sequence competes for p53 binding regardless of the nature of the DNA ends (data not shown). This result confirms that the nature of the DNA ends rather than any internal sequence appears to influence Ku binding.

The Order of Addition of the Oligonucleotides and Ku in a Competitive EMSA Is Critical to the Demonstration of Apparent Sequence Specificity

The above experiment (Fig. 2) clearly shows that the nature of the DNA ends affects the binding of Ku to DNA, yet all the reports in the literature claim that the end binding property of Ku shows no specificity for the nature of the DNA ends (14–17). A major difference between the previous reports and our studies was the order of addition of the competitor DNA and Ku in the EMSA. Fig. 3 shows just how critical the order of addition of the DNA and Ku is to the result of the assay. Oligonucleotides that differed only in their DNA end sequence (Table I) were used as competitor DNA against the TL4 radiolabeled oligonucleotide in an EMSA. When the probe and competitor DNA were added to the assay before Ku, all oligonucleotides competed for Ku binding to the TL4 probe regardless of their end structure (Fig. 3, lanes 8–13). This assay result supports those in the literature saying that Ku exhibits no specificity for any particular DNA end sequence. However, when the competitor DNA was added to the assay after the labeled DNA and Ku,
Transfer of Ku between DNA Molecules with Homologous Ends

Although Ku does not appear to exhibit any specificity for different DNA end sequences (Refs. 14, 15, and 17 and Fig. 3), the nature of the ends of the competitor DNA clearly influences its ability to bind Ku that is already bound to another (labeled) DNA molecule (Fig. 3). Only the TL4 competitor DNA could compete for Ku bound to the TL4-labeled DNA (Fig. 3, lanes 2–7). This suggested that only DNA with the same ends as the labeled DNA could compete for Ku binding or that only TL4 could be a successful competitor. This was tested by prebinding Ku to a labeled oligonucleotide with different DNA ends (TL3OHS; see Table I) and asking whether TL3OHS or TL4 would compete for Ku binding. Fig. 4 clearly shows that only TL3OHS competitor DNA could remove Ku from the labeled DNA and that TL4 had no effect. This supported the idea that only DNA with the same DNA ends as the labeled DNA competes for Ku. However, when another oligonucleotide, TLNC (see Table I), was used as the labeled probe, no DNA, including TLNC, could compete for Ku already bound to the probe (data not shown). The ends of these different oligonucleotides revealed that the ends of TL4 and TL3OHS were self-complementary, whereas those of TLNC were not; i.e., competition was only seen when the competitor DNA had ends complementary to the labeled DNA. This striking result explains the apparent sequence specificity of Ku DNA binding seen in our initial competition studies (Fig. 1).

Possible Mechanisms for the Transfer of Ku Between Two DNA Molecules

Two models (Fig. 5) are proposed to explain the phenomenon that once Ku has bound to one DNA molecule, another DNA molecule can only compete for Ku if its ends are complementary to those of the first DNA molecule.

The Two-site Model—This model assumes that Ku has two DNA binding sites and can transiently bind two DNA molecules, with the DNA bound to one site influencing which DNA molecule can bind in the other site.

Sliding Model—A transient coupling of the labeled DNA and the competitor DNA, through their complementary ends, enables Ku to slide from the probe DNA to the competitor oligonucleotide, thus resulting in apparent “competition.”

Both these models predict that in the presence of competitor DNA with ends complementary to the labeled DNA, Ku transfers directly from the labeled DNA to the competitor DNA without being released into solution first. This hypothesis was tested by incubating Ku with unlabeled DNA before adding labeled competitor DNA and monitoring the formation of Ku-competitor DNA complexes by EMSA. When Ku was bound to unlabeled TL4 DNA and challenged with labeled TL4 (complementary ends), a Ku-competitor DNA complex was observed (Fig. 6, lanes 1–7). No bands were seen when the competitor DNA (TLNC) with noncomplementary ends to the first DNA molecule was used (Fig. 6, lanes 8–15), confirming that the bands seen in Fig. 6, lanes 1–7, were not just due to competitor DNA binding excess Ku free in solution. Neither competitor DNA (TL4 or TLNC) resulted in the appearance of a Ku-DNA complex when TLNC was used as the unlabeled, first DNA molecule (Fig. 6, lanes 16 and 17); neither competitor DNA had ends complementary to TLNC (Table I). Together, these results show that Ku does transfer to the competitor DNA molecule provided that the DNA ends of the competitor oligonucleotide and the oligonucleotide that Ku is prebound to are complementary.

To determine whether Ku moves directly from the first DNA molecule to the competitor DNA or whether it is released into
solution before binding the competitor DNA, the labeled competitor DNA was added to the reaction in the presence of a 25 mM excess of unlabeled TLNC (noncomplementary) DNA. If the complementary competitor DNA (TL4) initially displaced Ku from the first DNA molecule into solution, then this Ku would be preferentially bound by the unlabeled DNA because it is in such excess over the labeled DNA (Fig. 3, lane 13). This would result in a reduction of the intensity of the Ku-competitor DNA band. However, adding the excess unlabeled competitor DNA had no effect on the binding of Ku to the labeled competitor DNA (Fig. 6, compare lanes 18 and 19), which suggests that Ku moves directly from one DNA molecule onto another without dissociation of Ku from the DNA (46).

The Extent of DNA End Complementarity Required for Ku Transfer

The data above (Figs. 2 and 4) show that Ku can transfer directly from one DNA molecule to another if the two oligonucleotides have complementary DNA ends. To determine the extent of complementarity required for this phenomenon, a series of double-stranded oligonucleotides, which differed only at their ends (Table I), were used as competitor DNA in gel shift assays in which TL4 was used as the labeled DNA. The results are summarized in Table I. Exact (100%) complementarity is not necessary to affect transfer of Ku. Ku efficiently transferred from TL4 (which has a 4-nucleotide overhang) to the TL5 oligonucleotide (which has a 5-nucleotide overhang at each end) (Table I) despite the presence of a non-base-paired nucleotide (Fig. 7a). Interestingly, Ku did not transfer from TL4 to TL3, an oligonucleotide with a 3-nucleotide overhang, despite both oligonucleotides having partially complementary ends (Table I and Fig. 7b). This lack of transfer could reflect the possible DNA “gap” present in the TL3-TL4 end complex (Fig. 7b). However, Ku could transfer from TL4 to oligonucleotides TL14, TL15, and TL16, albeit with reduced efficiency (Table I), despite the fact that the complexes TL4 would form with these oligonucleotides could also have DNA gaps (Fig. 7, c and d). Furthermore, when TL3 was used as both the labeled probe and the competitor DNA (such that there was no possibility of DNA gaps when the two pieces of DNA coupled), Ku transfer was still not seen (data not shown). This suggests that a complex between DNA ends with only three complementary nucleotides may not be sufficiently stable at 4 °C to allow Ku to move from one DNA molecule to the other and that a minimum of four complementary nucleotides is required.

The Activity of Multimeric Ku-DNA Complexes in DNA Competition Assays

Several groups (8, 15, 17, 40) have reported that multiple Ku molecules can bind to the same DNA molecule like beads on a string to give multimeric Ku-DNA complexes, with the number of Ku molecules binding being proportional to the length of the DNA. In agreement with these reports, titrating Ku against a fixed concentration of labeled DNA resulted in a ladder of progressively retarded bands (Fig. 8A). When labeled TL4 DNA (Fig. 8A) or TLB/A DNA (data not shown) was used, three complexes were observed, Ku(1), Ku(2), and Ku(3), with Ku(1) being the fastest migrating complex. The second band, Ku(2), did not appear until there was very little free DNA remaining. The third, most retarded band, Ku(3), was never as pronounced as the other two. The simplest interpretation of Fig. 8A is that Ku(1), Ku(2), and Ku(3) represent 1, 2, and 3 Ku units, respectively, binding to the same DNA molecule.

The experiments described so far in this article focused on the Ku(1) complex and established that Ku bound to one DNA molecule (in a Ku(1) complex) could transfer to a second DNA molecule only if the two oligonucleotides had complementary ends. To determine whether the same was true for multimeric Ku-DNA complexes, a similar Ku transfer gel shift assay was performed in which Ku was bound to the TL4 probe to give all

A TWO SITE MODEL

B SLIDING MODEL

Fig. 5. Two models to explain why only oligonucleotides with ends complementary to those of the DNA probe can compete for Ku binding. Arrow, direction of Ku movement.
three Ku complexes, Ku(1), Ku(2), and Ku(3), before competitor oligonucleotides with ends complementary (TL4) or noncomplementary (TLNC; see Table I) to the probe were titrated into the reaction (Fig. 8 B). The “complementary” competitor “competed” for Ku bound in all three Ku-DNA complexes. The disappearance of the Ku(2) complex coincided with an increase in the Ku(1) complex before this too was competed away. The “noncomplementary” competitor TLNC, even at very low concentrations, also competed for Ku in the Ku(3) and Ku(2) complexes, again resulting in an increase in the Ku(1) complex (Fig. 8 B). Conversely, TLNC did not compete for Ku in the Ku(1) complex. Furthermore, the Ku(2) complex was never fully lost even with a 500 M excess of TLNC. Since a noncomplementary competitor DNA could compete for Ku in Ku(2) and Ku(3) complexes but not in a Ku(1) complex, it suggests that Ku in the higher multimeric DNA complexes (Ku(2) and Ku(3)) is not equivalent to Ku in the Ku(1) complex; Ku in the higher multimeric DNA complexes binds DNA in a different manner from Ku in the Ku(1) complex. This result also explains why no Ku(2) or Ku(3) complexes are found until all the DNA is bound by Ku in the Ku(1) form; any free oligonucleotide would act as competitor DNA, and Ku(2) would dissociate to a Ku(1) complex.

Ku Can Form Different Interactions with DNA

The results from the Ku transfer assay (Fig. 8 B) show that the Ku-DNA interaction of at least some of the Ku molecules in a multimeric Ku-DNA complex, e.g. Ku(2), is not the same as that found in the Ku(1) complex. Thermal stability studies support this notion that Ku in the higher multimeric DNA complexes is not equivalent to Ku in the Ku(1) complex. The Ku(1) complex dissociated at 55 °C, whereas the Ku(2) complex had mostly dissociated into a Ku(1) complex by 45 °C (data not shown). In both the transfer EMSA (Fig. 8 B) and the thermal stability study, a decrease in Ku(2) coincides with an increase in Ku(1). This increase in Ku(1), and the fact very little free DNA is seen, suggests that only one Ku unit, rather than both units, leaves the Ku(2) complex. This suggests that one Ku unit in the Ku(2) complex is more susceptible to competitor DNA or heat denaturation than the other; i.e. the Ku units in the Ku(2) complex are not equivalent.

Further evidence that the Ku units in the Ku(2) complex were not equivalent came from differentiating between the two Ku units and asking which one left the Ku(2) complex in the presence of competitor DNA. Competitor DNA with ends noncomplementary to those of the probe was used so that only one Ku molecule rather than both molecules would leave the Ku(2) complex (see Fig. 8 B). The two Ku units in Ku(2) were differentiated by having one Ku molecule biotinylated and the other unlabeled. The biotinylated Ku bound DNA and migrated with the same mobility as unlabeled Ku (Fig. 9, compare lanes 1 and 3). The vast majority of Ku in the biotinylation reaction was labeled, since it all supershifted in the presence of streptavidin (Fig. 9, lanes 1 and 3). The vast majority of Ku in the biotinylation reaction was labeled, since it all supershifted in the presence of streptavidin (Fig. 9, lanes 1 and 3). The vast majority of Ku in the biotinylation reaction was labeled, since it all supershifted in the presence of streptavidin (Fig. 9, lanes 1 and 3). The vast majority of Ku in the biotinylation reaction was labeled, since it all supershifted in the presence of streptavidin (Fig. 9, lanes 1 and 3). The vast majority of Ku in the biotinylation reaction was labeled, since it all supershifted in the presence of streptavidin (Fig. 9, lanes 1 and 3). The vast majority of Ku in the biotinylation reaction was labeled, since it all supershifted in the presence of streptavidin (Fig. 9, lanes 1 and 3). The vast majority of Ku in the biotinylation reaction was labeled, since it all supershifted in the presence of streptavidin (Fig. 9, lanes 1 and 3). The vast majority of Ku in the biotinylation reaction was labeled, since it all supershifted in the presence of streptavidin (Fig. 9, lanes 1 and 3). The vast majority of Ku in the biotinylation reaction was labeled, since it all supershifted in the presence of streptavidin (Fig. 9, lanes 1 and 3).
biotinylated Ku was added first followed by more biotinylated Ku, all the Ku that remained bound to the probe DNA was supershifted by streptavidin (Fig. 9, lanes 11–13), whereas when nonlabeled Ku was added first, followed by more nonlabeled Ku, none of the Ku that remained bound to the probe after the addition of competitor DNA reacted with streptavidin (Fig. 9, lanes 14–16).

These striking results show that it is always the first Ku molecule that binds DNA in forming a Ku(2) complex that leaves the complex in the presence of nonhomologous DNA; the system shows a “first in, first out” rule. This confirms that the two Ku molecules interact with DNA in a distinct way. Furthermore, no equilibrium is reached between the first Ku molecule to bind the DNA and the second Ku molecule in the Ku(2) complex. In a sense, the second Ku molecule binding to the DNA displaces and weakens the binding of the resident Ku molecule. Again this strongly suggests that the Ku molecules in a Ku(2) complex do not bind DNA in the same way as each other.

**FIG. 8. The interaction of multiple Ku with a linear oligonucleotide.** A, 2–3 ng of the radiolabeled TL4 oligonucleotide was incubated with increasing concentrations of purified Ku (1.75, 3.5, 7, 10.5, 14, 21, 28, 35, 70, and 140 ng). Arrows indicate different Ku-DNA complexes. B, all three Ku-DNA complexes (Ku(1), Ku(2) and Ku(3)) were formed together by incubating 126 ng of Ku with 2–3 ng of the radiolabeled TL4 oligonucleotide. Various amounts (4, 8, 16, 32, 64, 128, 256, 400, 800, 1600, and 2000 ng) of unlabeled competitor DNA with ends complementary (TL4, lanes 2–12) or noncomplementary (TLNC, lanes 13–23) to the labeled probe were added. Lane 1 contains no competitor DNA.

**DISCUSSION**

The Ku protein first identified as a human nuclear autoantigen is now recognized as a highly conserved DNA end-binding protein consisting of two subunits, Ku80 and Ku70 (80 and 70 kDa, respectively) (1, 2). The Ku protein interacts with the catalytic subunit of the DNA-dependent kinase to form the activated kinase (3–5). All three subunits of the kinase are required for ds DNA break repair and V(D)J recombination (5, 28–33). The catalytic subunit of the kinase by itself cannot bind DNA, and the specific DNA binding requirements of repair and recombination are provided by the Ku component (3, 4, 41).

This makes study of the interactions of Ku with DNA of particular interest. In this study we have shown that Ku can bind to DNA in two different ways and that Ku can transfer directly from one DNA molecule to another.

**Ku Requires DNA with Complementary Ends to Transfer from One DNA Molecule to Another**—We have discovered that when Ku is bound to DNA in one form (Ku(1)) it can transfer from one DNA molecule to another but only if the two DNA molecules have complementary ends. We proposed two models to explain how this transfer of Ku may occur. The sliding model suggests that the two DNA molecules form a transient association through their complementary ends, and Ku simply slides from one oligonucleotide to the other. The sliding of Ku along DNA has also been suggested, but not proven, by other groups (8, 15), but very little is known about a possible mechanism of sliding. Our other model, the two-site model, suggests that Ku has two DNA binding sites and can transiently bind two pieces of DNA. With this model, to transfer from one oligonucleotide to another, Ku may release the DNA from one binding site and remain bound to the other DNA molecule. Alternatively, once both DNA binding sites are occupied, Ku may slide along the DNA away from the DNA junction, which then becomes unstable, and the two pieces of DNA dissociate. The difference between this and the sliding model is whether the coupling of the two DNA molecules requires Ku (two-site model) or is totally independent of Ku (sliding model). Regardless of the exact mechanism, transfer of Ku directly from one DNA molecule to another means that at some point during this transfer Ku is binding both DNA molecules simultaneously; i.e., Ku would be bridging two DNA ends. This bringing of DNA ends together is fundamental to both recombination and DNA double-strand break (dsb) repair. In vivo, Ku is likely to be complexed to other proteins, and one can postulate that Ku targets these proteins...
to regions of the DNA that require the joining of DNA ends. Ku may transfer these proteins across DNA breaks or nicks. Alternatively, these proteins may prevent Ku completely transferring from one DNA molecule to another and instead stabilize Ku in the position where it is bridging two DNA ends. In this way, Ku could hold the DNA ends together while other proteins function to ligate the two ends. The fact that a minimum of 4 complementary nucleotides between the ends of the two oligonucleotides is required to achieve transfer of Ku from one oligonucleotide to the other may imply that Ku promotes the joining of homologous DNA ends. In support of this speculation, Bryant and Johnston (42) have shown that cohesive-ended dsbs, which are thought to be the major type of dsb induced by radiation, are much less clastogenic than blunt-ended dsbs. This suggests that cohesive-ended dsbs are repaired more readily than blunt-ended dsbs, which says that recognition of homologous DNA ends in vivo is an important step in dsb repair. Exact complementarity between the two DNA ends is not essential for Ku transfer; a gap of one nucleotide or a protrusion of one nucleotide, as well as a DNA nick, can be tolerated. This could be significant given that such perturbations in DNA structure are likely to be found when DNA ends are joined during double strand break repair and V(D)J recombination (43, 44), both processes that involve Ku.

Artifact of Sequence Specificity of Ku Binding—One consequence of the clear requirement we have shown for complementary ends in the transfer of the Ku(1) complex from one DNA molecule to another is the striking artifact of sequence-specific DNA binding this phenomenon can produce. Thus, all studies that detect sequence-specific DNA binding by Ku need to carefully exclude this phenomenon as the basis for the apparent specificity of Ku. The lack of sequence specificity in our case was established by experiments that demonstrated: (a) the critical importance of the order of addition of the oligonucleotides and Ku in a competitive EMSA; (b) that Ku did not recognize the sequence within a ds closed circular plasmid; and (c) that changing the DNA ends of an oligonucleotide changed its ability to apparently bind Ku in a sequence-specific manner. Therefore, as a minimum requirement to show sequence specificity using a competitive EMSA, any study should observe two rules: (a) ensure that the competitor DNA and probe DNA are added to the reaction before Ku; and (b) the ability of Ku to recognize the proposed specific sequence in the context of a closed circular DNA should be established, as in the recent study by Giffin et al. (27). Footprint studies have also been used to show sequence-specific binding of Ku (22, 24). Given that Ku binds nonspecifically to any DNA with a free end, it is important to have nonspecific DNA present during the footprint reaction. Once again the order of addition of the DNA and Ku is important; thus rule (a) above also applies to footprinting assays. More importantly, a second criteria to be met with these assays is to demonstrate that mutating the sequence in question alters the Ku footprint. This is particularly important with Ku since it is postulated to slide along the DNA (8, 15) and, therefore, the conditions of the footprint assay (e.g. DNA: protein ratio, time and temperature of incubation, and DNA length) may influence where Ku binds on the DNA.

Even when all these stringent criteria are met, any sequence specificity observed may not be an inherent property of the Ku protein. Several studies have suggested that contaminating proteins, in what in some cases appeared to be a homogeneous preparation of Ku, confer sequence specificity on Ku. Generisch et al. (38) propose that the specific binding of Ku to the promoter region of the human collagen type IV genes is a result of contamination of the purified Ku preparation with the TATA-binding protein. Similarly, de Vries et al. (15) suggested that the presence of nuclear factor I was responsible for the specific binding of Ku (or nuclear factor IV) to the adenovirus type 2 origin of replication, whereas the presence of the TRAC protein may confer transferrin receptor transcriptional control element specificity on Ku (23). The interaction of different proteins with Ku could thus alter the specificity of Ku binding to DNA, which would explain why Ku apparently specifically recognizes four unrelated DNA sequences; the octamer motif (22), the transferrin receptor transcriptional control element and proximal sequence element (23, 24), the rDNA promoter (25, 26, 45), and the negative regulatory element 1 sequence in the long terminal repeat of the mouse mammary tumor virus (27).

Ku Interacts with DNA in Two Different Ways—When higher concentrations of Ku were added to the gel mobility shift assay, a ladder of three progressively retarded bands was observed, which is in agreement with other groups who have reported that multiple Ku molecules can bind to the same DNA molecule like beads on a string to give multimeric Ku-DNA complexes (8, 15, 17, 40). However, the reported footprint of Ku plus the spacer (the distance between neighboring Ku units) in such multimeric Ku-DNA complexes is 27–30 base pairs (15), which suggests that the short 34-base pair oligonucleotide used in our study should only bind one Ku binding unit rather than three as we observed. This discrepancy suggested that at high Ku: DNA ratios, the interaction of Ku with short DNA fragments is different from its interaction with longer DNA, in which the beads on a string structure is seen. This was the first indication that Ku may bind DNA in different ways. Evidence to support this idea was that Ku in a multimeric DNA complex (Ku(2) and Ku(3)) could transfer from one DNA molecule to another regardless of the nature of the DNA ends; in contrast, Ku in the monomeric DNA complex (Ku(1)) required DNA with complementary ends to be able to transfer between DNA molecules. Since the type of Ku-DNA complex formed is dependent on the relative concentrations of Ku and DNA, the specificity of recombination and double strand break repair may be critically controlled by the local Ku concentration, with the Ku(1) form promoting homologous recognition, whereas the Ku(2) form is more promiscuous.

By differentially labeling the two Ku binding units in the Ku(2) complex, we showed that the first Ku to bind the DNA in forming the Ku(2) complex is always the first Ku to leave the complex in the presence of competitor DNA. This confirms the Ku molecules in the Ku(2) complex are not equivalent and that there are different Ku-DNA interactions within the Ku(2) complex. Moreover, this result also says that within the Ku(2) complex there is no interchange between the two forms of Ku; once a Ku(2) complex has formed, a Ku unit bound to DNA in one form cannot change and become bound in the other form. The simplest interpretation of the two forms of Ku binding found in the Ku(2) complex is that one Ku molecule is bound to an internal portion of the oligonucleotide, whereas the other Ku molecule is bound to the end of the DNA. The fact that these two Ku-DNA interactions are distinguishable is important because it says that Ku has different properties depending on how it is bound to DNA. This may be significant in influencing the function of Ku, for example, whether Ku acts in transcription or whether it is involved in bringing DNA ends together in dsb repair. Moreover, since Ku can form two different DNA interactions, it is possible that any Ku-interacting proteins may exhibit a specificity for Ku bound to DNA in one form over Ku bound in the other form. The difficulty to date in finding partner proteins of Ku involved in dsb repair may be a reflection that the wrong Ku-DNA complex is being used to search for such proteins. It will be interesting to use both types of Ku-DNA interaction discussed in this study to look for new
proteins that interact with Ku since the discovery of other Ku-interacting proteins will be important to understand the mechanism of action of Ku.

Acknowledgments—We thank Kathryn Ball, Robin Fåhraeus, Ted Hupp, David Lilley, and Neil Perkins for critical reading of the manuscript and for helpful discussions. We also thank Byron Hann and Dimitry Goldgaber for suggestions.

REFERENCES
1. Yaneva, M., Ochs, R., McRorie, D. K., Zweig, S., and Busch, H. (1985) Biochim. Biophys. Acta 841, 22–29
2. Mimori, T., Hardin, J. A., and Steitz, J. A. (1986) J. Biol. Chem. 261, 2274–2278
3. Dvir, A., Stein, L. Y., Calore, B. L., and Dynan, W. S. (1993) J. Biol. Chem. 268, 10440–10447
4. Gottlieb, T. M., Gottlieb, J. A., and Steitz, J. A. (1986) J. Biol. Chem. 261, 10546–10552
5. Blunt, T., Finnie, N. J., Taccioli, G. E., Smith, G. C., Demengeot, J., Gottlieb, T. M., Mizuta, R., Varghese, A. J., Alt, F. W., Jeggo, P. A., and Jackson, S. P. (1995) Cell 80, 813–823
6. Mimori, T., Akizuki, M., Yamagata, H., Inada, S., Yoshida, S., and Homma, M. (1994) J. Mol. Biol. 231, 331–338
7. Blier, P. R., Griffith, A. J., Craft, J., and Hardin, J. A. (1992) J. Biol. Chem. 267, 325–338
8. Falzon, M., Fewell, J. W., and Kuff, E. L. (1993) J. Biol. Chem. 268, 10546–10552
9. Bardwell, J. A., Bardwell, L., Tomkinson, A. E., and Friedberg, E. C. (1994) Science 265, 2082–2085
10. Bryant, P. E., and Johnston, P. J. (1993) Mutat. Res. 290, 289–296
11. Bryant, P. E., and Johnston, P. J. (1993) Mutat. Res. 290, 289–296
12. Price, A. (1993) Semin. Cancer Biol. 4, 61–71
13. Bryant, P. E., and Johnston, P. J. (1993) Mutat. Res. 290, 289–296
14. Bryant, P. E., Johnston, P. J. (1993) Mutat. Res. 290, 289–296
15. Bryant, P. E., Johnston, P. J. (1993) Mutat. Res. 290, 289–296
16. Price, A. (1993) Trends Biochem. Sci. 20, 412–415
17. Kuhn, A., Stefanovsky, V., and Grummt, I. (1993) Nucleic Acids Res. 21, 2057–2063
18. Zhang, W. W., and Yaneva, M. (1992) Biochem. Biophys. Res. Commun. 186, 574–579
19. Kuno, T., and Kusumoto, K. (1992) Biochem. Biophys. Res. Commun. 186, 574–579
20. Jeltsch, A., and Fotedar, A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2683–2689
21. May, G., Sutton, C., and Gould, H. (1991) J. Biol. Chem. 266, 3052–3059
22. Roberts, M. R., Han, Y., Finberg, A., Hui, L., and Ruddle, F. H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6354–6358
23. Kuno, T., and Kusumoto, K. (1992) Biochem. Biophys. Res. Commun. 186, 574–579
24. Kuno, T., and Kusumoto, K. (1992) Biochem. Biophys. Res. Commun. 186, 574–579
25. Hoff, C. M., Ghosh, A. K., Prabakhar, B. S., and Jacob, S. T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 762–766
26. Niu, H., and Jacob, S. T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9101–9105
27. Giffin, W., Torrance, H., Rodda, D. J., Prefontaine, G. G., Pope, L., and Hache, R. (1996) Nature 380, 265–268
28. Getts, R. C., and Stamos, T. D. (1994) J. Biol. Chem. 269, 15981–15984
29. Rathmell, W. K., and Chu, G. (1994) Mol. Cell. Biol. 14, 4741–4748
30. Taccioli, G. E., Gottlieb, T. M., Blunt, T., Priestley, A., Demengeot, J., Mizuta, R., Lehmann, A. R., Alt, F. W., Jackson, S. P., and Jeggo, P. A. (1994) Science 265, 1442–1445
31. Kirchgeissner, C. U., Patil, C. K., Evans, J. W., Cuomo, C. A., Fried, L. M., Carter, T., Oettinger, M. A., and Brown, J. M. (1995) Science 267, 1178–1183
32. Lees-Miller, S. P., Godbout, R., Chen, D. W., Weinfeld, M., Day, R. S., Barron, G. M., and Allalunisturner, J. (1995) Science 267, 1183–1185
33. Sigley, J. D., Menninger, J. C., Hartley, K. O., Ward, D. C., Jackson, S. P., and Anderson, C. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7515–7519
34. Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. (1992) Cell 71, 875–886
35. Kadonaga, J. T., and Tjian, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 83, 5889–5893
36. el Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992) Nat. Genet. 1, 45–49
37. Funk, W. D., Pak, D. T., Karas, R. H., Wright, W. E., and Shay, J. W. (1992) Mol. Cell. Biol. 12, 2866–2871
38. Genersch, E., Eckerskorn, C., Lottspeich, F., Herzog, C., Kuhn, K., and Posch, E. (1995) EMBO J. 14, 791–800
39. Tosh, E. C., Marusic, L., Ochsen, A., Pathy, A., Pongor, S., Giacca, M., and Falaschi, A. (1993) Nucleic Acids Res. 21, 3257–3263
40. Zhang, W. W., and Yaneva, M. (1992) Biochem. Biophys. Res. Commun. 186, 574–579
41. Morozov, V. E., Falzon, M., Anderson, C. W., and Kuff, E. L. (1994) J. Biol. Chem. 269, 16684–16688
42. Bryant, P. E., and Johnston, P. J. (1993) Mutat. Res. 290, 289–296
43. Price, A. (1993) Semin. Cancer Biol. 4, 61–71
44. Jackson, S. P., and Jeggo, P. A. (1995) Trends Biochem. Sci. 20, 412–415
45. Kuhn, A., Stefanovsky, V., and Grummt, I. (1993) Nucleic Acids Res. 21, 2057–2063
46. Berg, O. G., Winter, R. B., and von Hippel, P. H. (1981) Biochemistry. 20, 6929–6948