

# DNA-dependent protein kinase: DNA binding and activation in the absence of Ku

(DNA repair/V(D)J recombination/ionizing radiation)

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**ABSTRACT** In mammalian cells, double-strand break repair and V(D)J recombination require DNA-dependent protein kinase (DNA-PK), a serine/threonine kinase that is activated by DNA. DNA-PK consists of a 460-kDa subunit (p460) that contains a putative kinase domain and a heterodimeric subunit (Ku) that binds to double-stranded DNA ends. Previous reports suggested that the activation of DNA-PK requires the binding of Ku to DNA. To investigate this further, p460 and Ku were purified separately to homogeneity. Surprisingly, p460 was capable of binding to DNA in the absence of Ku. The binding of p460 to double-stranded DNA ends was salt-labile and could be disrupted by single-stranded or supercoiled DNA, properties distinct from the binding of Ku to DNA. Under low salt conditions, which permitted the binding of p460 to DNA ends, the kinase was activated. Under higher salt conditions, which inhibited the binding of p460, activation of the kinase required the addition of Ku. Significantly, when the length of DNA decreased to 22 bp, Ku competed with p460 for DNA binding and inhibited kinase activity. These data demonstrate that p460 is a self-contained kinase that is activated by direct interaction with double-stranded DNA and that the role of Ku is to stabilize the binding of p460 to DNA ends.

A DNA double-strand break (DSB) disrupts the integrity of DNA so that neither strand can serve as a template for repair of the lesion. DSBs are induced by ionizing radiation or created by cleavage mechanisms that initiate meiotic recombination (1) or V(D)J recombination (2). The repair of DSBs is accomplished either by homologous recombination or by nonhomologous end-joining (3). In mammalian cells, DSB repair proceeds predominantly by nonhomologous end joining. Four complementation groups of mutant rodent cells are hypersensitive to ionizing radiation and defective in V(D)J recombination, the site-specific recombination pathway that generates diversity in the Ig and T cell receptor genes (2). Three of the corresponding genes encode components of DNA-dependent protein kinase (DNA-PK): p460, Ku70, and Ku86 (4–7). The fourth gene encodes XRCC4 (8), a 38-kDa protein that stimulates the activity of DNA ligase IV (9, 10).

DNA-PK has been defined in the literature as an enzyme with two components (11, 12). One is a subunit of 460 kDa (p460) that shares a domain of homology with members of the phosphatidylinositol 3-kinase family (13). The other is the Ku autoantigen, a heterodimer of 70 and 86 kDa (12). Ku binds to double-stranded DNA ends, nicks, and stem-loop structures (14–16) and is able to translocate along the DNA helix by an ATP-independent process (17). Although the *in vivo* substrates for DNA-PK remain undefined, *in vitro* experiments demon-

strate that the kinase can regulate its own activities by autophosphorylation of p460, Ku70, and Ku86. Autophosphorylation of p460 inactivates its kinase activity and causes its dissociation from the Ku–DNA complex (18). Autophosphorylation of Ku confers an ATPase activity to Ku (19), which has been reported to mediate a helicase activity (20).

Despite these advances, little is known about how DNA activates the kinase. Previous studies suggested that DNA-PK is activated only if Ku binds to DNA and recruits p460 to assemble the full DNA-PK complex (12). However, DNA-PK is not activated by several DNA substrates that bind Ku, including DNA hairpins and nicks (V. Smider, W. K. Rathmell, G. Brown, S. Lewis, and G.C., unpublished work) and cisplatin-cross-linked DNA (22). To examine this discrepancy in greater detail, we purified to virtual homogeneity p460 and Ku as separate preparations. With these preparations, we confirmed that Ku will stimulate the assembly of p460 on DNA ends and activation of the kinase. Surprisingly however, even in the absence of Ku, p460 was capable of binding to DNA and being activated. Thus, p460 contains a complete kinase domain that is activated by direct interaction with double-stranded DNA ends.

## MATERIALS AND METHODS

**Plasmids and Oligonucleotides.** The plasmid pBluescript II KS+ (Stratagene) was prepared by using a Qiagen maxi plasmid column (Qiagen, Chatsworth, CA). Supercoiled plasmid DNA was further purified by agarose gel electrophoresis in the presence of ethidium bromide followed by electroelution. Contaminating nicked plasmid DNA was estimated to be less than 1%. The sequences of the oligonucleotides used were as follows: 32-bp oligonucleotide, 5'-GGCCGCACGCGTC-CACCATGGGGTACAACACTAC-3'; 22-bp oligonucleotide, 5'-GATCTAGCTTCATGCACCCGGAC-3'.

The duplex DNA probes were made by annealing complementary oligonucleotides to the oligonucleotides above in a 1:1 molar ratio to form duplex DNA fragments with 4-base 5' overhanging ends that were labeled by incubation with [ $\alpha$ -<sup>32</sup>P]dCTP (6,000 Ci/mmol; 1 Ci = 37 GBq) and Klenow fragment of *Escherichia coli* DNA polymerase I. Poly(dA) and poly(dT) (Pharmacia) were stored at –20°C in TE buffer (10 mM Tris-HCl, pH 7.5/1 mM EDTA).

**Electrophoretic Mobility Shift Assay (EMSA).** DNA binding activity was measured by an EMSA (V. Smider, W. K. Rathmell, G. Brown, S. Lewis, and G.C., unpublished work and ref. 23). Binding reactions were performed by incubating 0.2 ng of <sup>32</sup>P-labeled f32 DNA with protein preparations in buffer B (10 mM Tris-HCl, pH 7.5/1 mM EDTA/5% glycerol/1 mM DTT) containing different concentrations of NaCl at 25°C for

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: EMSA, electrophoretic mobility shift assay; DNA-PK, DNA-dependent protein kinase.

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5 min. Protein was diluted to working concentrations in buffer B and always added last. When both p460 and Ku were present, p460 was added last. After incubation, the products of the binding reaction were resolved by electrophoresis at 10 V/cm through a 4% polyacrylamide gel in TGE buffer (50 mM Tris, pH 8.5/0.38 M glycine/2 mM EDTA). The gels were dried on Whatman 3MM paper and subjected to autoradiography.

**Protein Purification.** The Ku and p460 subunits of DNA-PK were purified from human placenta essentially as described (24), with modifications noted herein. All procedures were performed at 4°C, and all buffers were supplemented with 10 mM 2-mercaptoethanol and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride/2 mM benzamidine/0.7 μM leupeptin/2 μM pepstatin A). Ku and p460 were monitored during fractionation by the EMSA and SDS/PAGE.

Extract was made from 250 g of a frozen human placenta and fractionated by DEAE-fast flow chromatography as described (24). The eluate from the DEAE column was dialyzed against buffer A (50 mM Tris-HCl, pH 7.5/5% glycerol/0.1 M NaCl/5 mM EDTA) and applied to a 50-ml heparin-agarose column. Bound proteins were step-eluted with buffer A containing 1 M NaCl. The eluate was dialyzed with buffer A and bound to a 7-ml column of DNA-cellulose (Sigma). The p460 and a small proportion of the bound Ku were eluted with a 0.1–0.4 M NaCl gradient in buffer A, whereas the majority of Ku was subsequently step-eluted in 1 M NaCl. Fractions containing p460 were pooled, adjusted to 0.1 M NaCl, and applied to an oligonucleotide affinity column consisting of 1 ml of NHS (*N*-hydroxy succinimide)-agarose (HiTrap, Pharmacia) coupled to 0.8 mg of f32 according to the manufacturer's protocol. One oligonucleotide in the double-stranded f32 was substituted at the 3' end with an amino group to allow efficient coupling to the NHS-agarose resin (25). The oligonucleotide column was developed with a 0.1–0.5 M NaCl gradient in buffer A. Under these conditions, p460 eluted at 0.25–0.30 M NaCl, and the majority of Ku remained bound to the resin, because Ku was normally eluted at 0.55–0.60 M NaCl. The trace amount of Ku contaminating the p460 fraction was removed by running the pooled p460 fraction through the oligonucleotide affinity column at 0.25 M NaCl. Under these conditions, p460 was collected in the flow-through, whereas the contaminating Ku and a minor fraction of p460 remained bound to the resin. The flow-through was adjusted to 0.1 M NaCl and subjected to Mono Q FPLC as described (24), except without addition of Tween 20. Fractions containing Ku from the DNA-cellulose column were adjusted to 0.1 M NaCl and

bound to the oligonucleotide column. The resin was washed with buffer A containing 0.35 M NaCl to remove trace amounts of p460. Ku was eluted at 1 M NaCl and subjected to Mono Q FPLC as described above. The Ku and the p460 preparations were judged by Coomassie blue staining of SDS gels to be greater than 95% pure (Fig. 1A). Active fractions were stored in aliquots at –80°C. Working solutions were stored in 0.5× buffer B with 50% glycerol/5 mM DTT/100 mM NaCl at –20°C for no more than 2 weeks. Each preparation yielded approximately 2 mg of Ku and 0.4 mg of p460.

**DNA-PK Assay.** Kinase assays were performed as described (26), with some modifications. Reactions were done in buffer C (10 mM Tris-HCl, pH 7.5/1 mM EDTA/10 mM MgCl<sub>2</sub>/0.25 mM ATP/5% glycerol/1 mM DTT). Unless otherwise indicated, each reaction contained 35 ng of p460, 11 ng of Ku (corresponding to a 1:1 molar ratio of p460:Ku), 5 μg of the specific peptide substrate (EPPLSQEAFADLWKK), and 1.25 μCi of [ $\gamma$ -<sup>32</sup>P]ATP in 5 μl. DNA and NaCl were added as indicated. Mixtures were incubated for 15 min at 30°C. Reactions were stopped by adding 5 μl of 30% acetic acid and then spotted onto phosphocellulose filters (Whatman P81). Filters were washed in four changes of 250 ml of 15% acetic acid. Radioactivity bound to the filters was measured by scintillation counting.

## RESULTS

**Purification of p460 Free of Ku Contamination.** The components of DNA-PK were purified from human placenta, with special care taken to separate p460 from Ku. With conventional chromatographic methods, however, we failed to obtain preparations of p460 devoid of Ku. Contamination with Ku was invariably found in the p460 fractions either by immunoblot or by Coomassie blue staining of overloaded SDS/PAGE gels. This contamination cofractionated with p460 over Mono Q, Mono S FPLC, and hydroxyapatite chromatography even under conditions designed to disrupt protein–protein interactions (0.2% Tween 20/0.01% Nonidet P-40/1.5 M NaCl).

However, it was possible to separate Ku from p460 by using an oligonucleotide column to which Ku bound with high affinity. When the purified p460 was resolved by SDS/PAGE and stained with Coomassie blue, no bands corresponding to Ku could be detected (Fig. 1A). To obtain a more sensitive estimate of contamination, the presence of Ku was determined by quantitative immunoblot. When 2.5 μg of the p460 preparation was immunoblotted with antiserum HT, which is

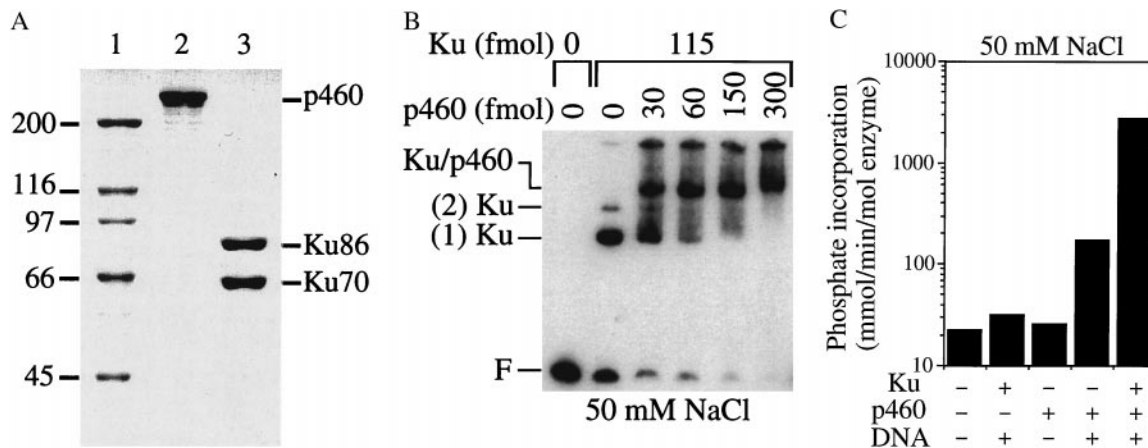


FIG. 1. Analysis of purified p460 and Ku. (A) SDS/PAGE. Purified p460 (lane 2, 350 ng) and Ku (lane 3, 350 ng) were resolved by SDS/PAGE in 8% gels and stained with Coomassie blue. Size markers (lane 1) are defined by their molecular masses in kilodaltons. (B) EMSA. Labeled f32 DNA (0.2 ng) was incubated with Ku (18 ng) and increasing amounts of p460 in 5 μl of buffer B containing 50 mM NaCl and analyzed by EMSA. F denotes the position of free probe. (C) Kinase activity. Peptide substrate (5 μg) was incubated with various combinations of Ku (11 ng), p460 (35 ng), and linear plasmid DNA (300 ng) in 5 μl of buffer C containing 50 mM NaCl, and kinase activity was determined.

specific for Ku70 (27), a weak band corresponding to Ku70 was detected. By comparing the contaminating band to a serial dilution of purified Ku loaded onto the same blot, the amount of contaminating Ku was estimated to be between 0.2 ng and 0.4 ng per 2.5  $\mu$ g of p460 (data not shown). Thus, the molar ratio of Ku to p460 in the purified p460 preparation was between 1:2,000 and 1:4,000.

To confirm that the purified p460 was fully active, it was tested for assembly into a complex with Ku on DNA by EMSA. When increasing amounts of p460 were added to fixed amounts of Ku and labeled f32 DNA, the Ku-containing complex shifted to a lower mobility complex (Fig. 1B). When p460 was added in a 1:1 molar ratio, virtually all of the Ku-f32 complex was shifted to a complex of f32 with Ku and p460, which were presumably assembled as DNA-PK.

The purified p460 was also tested for its kinase activity in a buffer containing 50 mM NaCl. In the absence of DNA, neither the p460 nor the Ku preparation had any detectable kinase activity. In the presence of linearized plasmid DNA, purified Ku had no kinase activity on its own. Surprisingly however, purified p460 showed a significant level of DNA-stimulated kinase activity (Fig. 1C). This kinase activity was further increased by 10-fold when Ku was added to p460 in an equimolar amount. The molar ratio of Ku to p460 that gave maximal kinase activity was approximately 1.3:1 (data not shown), in agreement with published observations (24). Therefore, in relatively low salt of 50 mM NaCl, p460 appeared to have a low level of DNA-stimulated kinase activity in the absence of Ku. This unexpected result suggested that p460 might be activated by binding directly to DNA.

**p460 and Ku Bind to DNA with Different Properties.** Purified p460 and Ku were tested for DNA binding by using EMSA. In low salt (10 mM NaCl) buffer, Ku bound to the labeled f32 DNA by forming two distinct complexes, repre-

senting one or two binding events (Fig. 2A). A mixture of Ku and p460 in a 1:1 molar ratio produced a distinct Ku-p460 complex. Surprisingly, p460 also bound to f32, forming a major complex that was retained in the well of the gel and a minor complex that migrated slightly faster in the gel than the Ku-p460 complex (Fig. 2A and data not shown). In fact, the DNA binding activity of p460 could not have been produced by the small amount of Ku in the p460 preparation, because 35 ng of p460 (76 fmol) shifted about 0.1 ng of f32 (5 fmol) and 35 ng of p460 contained less than 0.04 fmol of Ku (see above). Therefore, any Ku in the p460 preparation produced at most 0.8% of the observed binding activity. We conclude that a complex can form between p460 and f32 in the absence of Ku.

When poly(dT) was added to the binding reaction as a competitor, the binding of p460 to f32 was disrupted (Fig. 2A, lanes 6-10), whereas binding of Ku to f32 was unaffected (Fig. 2A, lanes 1-5). When poly(dT) was added to binding reactions containing both Ku and p460, the Ku-p460 complex with DNA was disrupted, leaving a residual complex of Ku with DNA (Fig. 2A, lanes 11-15). These results indicate that p460 recognizes single-stranded poly(dT) and double-stranded DNA ends, in marked contrast to Ku, which fails to recognize poly(dT) (23).

The disruption of DNA-PK by poly(dT) provided a second method for assessing the p460 preparation for Ku contamination. In a reconstruction experiment, Ku was deliberately mixed with the p460 preparation in a 1:100 molar ratio, and poly(dT) was added to disrupt the Ku-p460 complex (Fig. 2B). The small amount of admixed Ku was easily detected as a Ku complex (Fig. 2B, lanes 4 and 5). By contrast, no Ku complex could be detected in the p460 preparation, even after overexposure of the gel (Fig. 2B, lane 2). Thus, if Ku binding activity was present in the p460 preparation, it was much less than 1% of the observed binding activity.

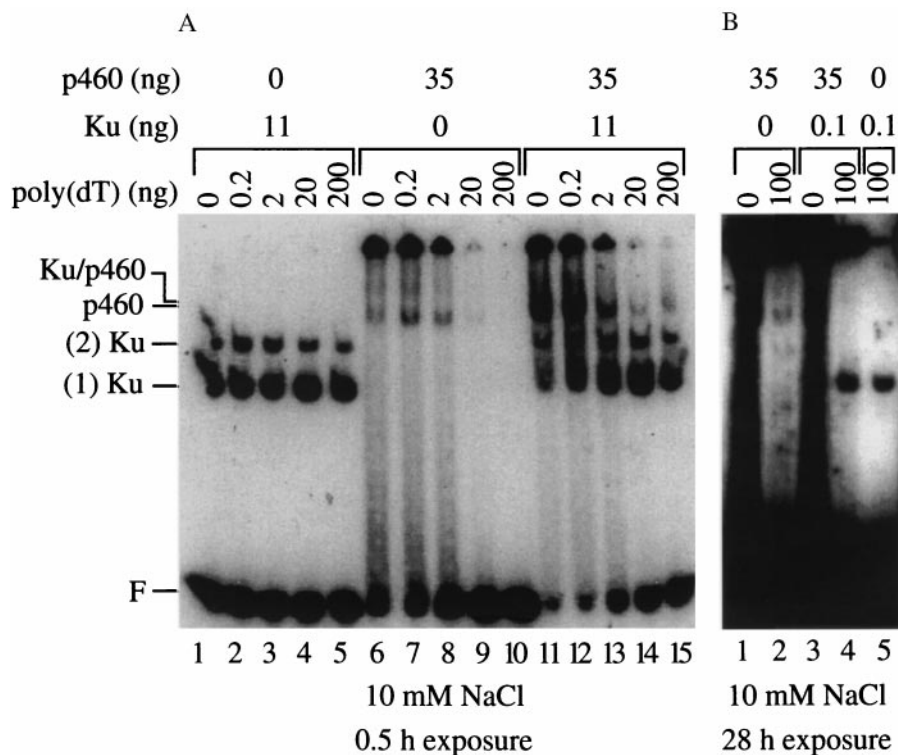


FIG. 2. DNA binding of Ku, p460, and the Ku-p460 complex. (A) DNA binds to Ku, p460, and Ku-p460 complexes in distinct complexes that are affected differently by poly(dT). Labeled f32 DNA (0.2 ng) and various amounts of poly(dT) were incubated with Ku (11 ng), p460 (35 ng), or an equimolar mixture of both in 5  $\mu$ l of buffer B containing 10 mM NaCl and analyzed by EMSA. Positions of individual complexes are indicated to the left of the gel. (B) Absence of Ku binding activity in the p460 preparation. Labeled f32 DNA (0.2 ng) with or without poly(dT) was incubated with Ku, p460, or a mixture of Ku and p460 (in a molar ratio of 1:100) in 5  $\mu$ l of buffer B containing 10 mM NaCl and analyzed by EMSA. Gels were exposed to x-ray film for the indicated times.

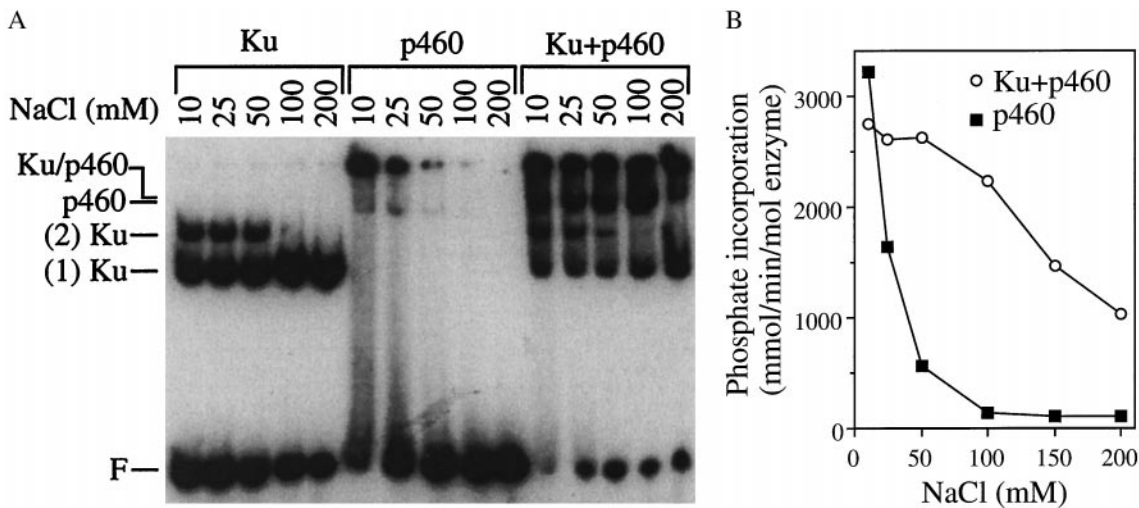


FIG. 3. Effect of salt on DNA binding and kinase activities of p460 and Ku-p460 complexes. (A) Increasing salt disrupts DNA binding of p460 but not of Ku. Labeled f32 DNA (0.2 ng) was incubated with Ku (11 ng), p460 (35 ng), or an equimolar mixture of both in 5  $\mu$ l of buffer B containing various concentrations of NaCl and analyzed by EMSA. (B) Increasing salt inhibits kinase activity strongly for p460 and weakly for Ku-p460 complexes. Kinase activities of p460 or the Ku-p460 complex were measured after stimulation by f32 DNA (0.2 ng) in buffer C containing various concentrations of NaCl by using the same protein concentrations as in A.

Binding of p460 to f32 DNA was disrupted with increasing NaCl concentration in the reaction buffer, whereas binding of Ku to f32 was largely unaffected (Fig. 3A). Thus, p460 binding to DNA is salt labile, another property that is markedly different from Ku and further evidence that the unique properties of the p460 DNA-binding activity were not due to Ku contamination.

Kinase activity was determined by using the same protein and DNA concentrations used for studying DNA binding activity. At low salt (10 mM NaCl), f32 activated either p460 or the full Ku-p460 complex, although surprisingly, kinase activity was slightly but reproducibly greater for p460 than for Ku-p460 (Fig. 3B). With increasing salt concentrations, the kinase activity of p460 fell sharply, becoming undetectable at 100 mM NaCl, but the activity of the Ku-p460 complex fell more slowly, remaining significant even at 200 mM NaCl. The reduction of p460 kinase activity paralleled the decrease in p460 DNA-binding activity with increasing salt concentration (Fig. 3 compare A and B). Therefore, the DNA-binding and kinase activities of p460 were equally sensitive to salt. Furthermore, the addition of Ku to p460 at 10 mM NaCl produced a measurable decrease in kinase activity, providing further evidence that the observed p460 kinase activity was Ku-independent.

**The p460 Kinase Is Activated Specifically by Double-Stranded DNA Ends.** The binding of p460 to f32 DNA was inhibited to the same extent by addition of supercoiled or linear plasmid DNA (data not shown), indicating that p460 bound to DNA with low selectivity for the DNA ends. To determine whether p460 kinase activity had a similar selectivity for DNA ends, we examined activation of the kinase with different forms of DNA: supercoiled plasmid, plasmid linearized with *Bam*HI, or plasmid digested to 15 fragments with *Dpn*I. Supercoiled plasmid failed to stimulate the kinase activity of p460, plasmid DNA linearized with *Bam*HI activated the kinase slightly at high DNA concentrations, and plasmid DNA digested with *Dpn*I activated the kinase strongly (Fig. 4).

The dramatically increased kinase activation with multiply cleaved compared with singly cleaved plasmid DNA was only partly explained by the increased number of DNA ends. In fact, when equivalent numbers of DNA ends were added for a series of DNA fragments from 700 bp to 32 bp, kinase activation increased with decreasing length (data not shown). Furthermore, the addition of either supercoiled or linearized plasmid

DNA inhibited activation of p460 kinase by f32 DNA (data not shown). Thus, efficient activation of purified p460 is compromised by binding of p460 to internal sites on DNA without concomitant activation.

When Ku was added to p460, linearized and *Dpn*I-cleaved plasmid DNA strongly activated the kinase but supercoiled plasmid DNA did not (Fig. 4). The levels of kinase activation were significantly higher for Ku plus p460 than for p460 alone. When equivalent numbers of DNA ends were added for DNA fragments from 700 bp to 32 bp, kinase activation no longer increased with decreasing length (data not shown). Thus, in the presence of Ku, activation of p460 is no longer compromised by internal sites on DNA.

The p460 kinase and the Ku-p460 complex were activated by the same spectrum of DNA ends, which included blunt ends, 5' overhanging ends, and dephosphorylated ends, but were not activated by single-stranded DNA in the form of poly(dA) or poly(dT) (data not shown). Therefore, the activation of p460 kinase required double-stranded DNA ends even in absence of Ku and despite the low selectivity of p460 for DNA ends.

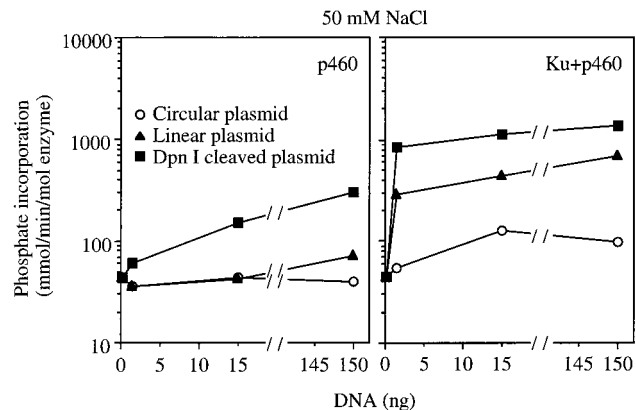


FIG. 4. p460 and the Ku-p460 complex are both specifically activated by DNA ends. Kinase activities of p460 or of Ku-p460 complexes were measured using different forms of plasmid DNA as substrate. Plasmid DNA was linearized with *Bam*HI, digested to 15 fragments with *Dpn*I, or left as supercoiled DNA and incubated with p460 (35 ng) with or without Ku (11 ng) in buffer C containing 50 mM NaCl.

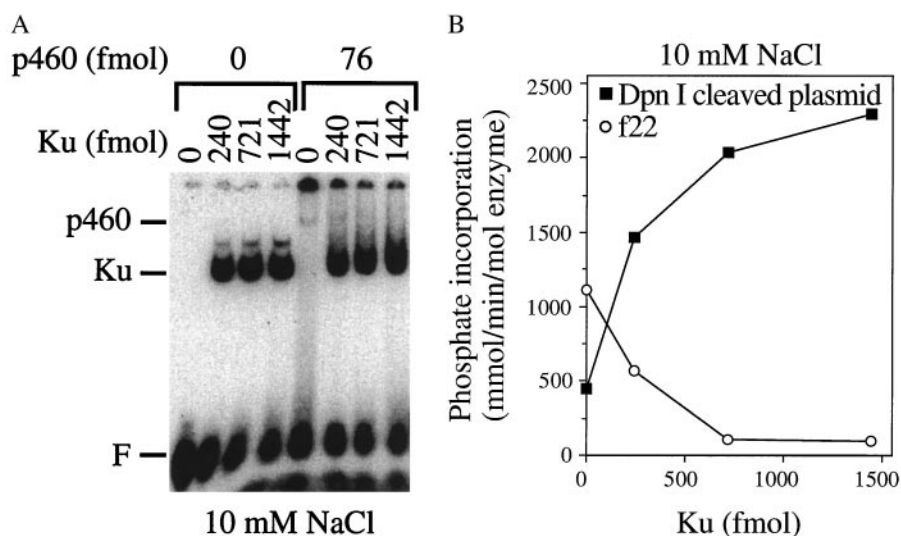


FIG. 5. DNA binding and kinase activation of p460 by f22 DNA are inhibited by the addition of Ku. (A) DNA binding. Labeled f22 DNA (1.4 ng, 100 fmol) was incubated with p460 (35 ng, 76 fmol) plus different amounts of Ku in 5  $\mu$ l of buffer B containing 10 mM NaCl and then analyzed by EMSA. (B) Kinase activation. Kinase activity was measured for p460 (76 fmol) with different amounts of Ku in 5  $\mu$ l of buffer C containing 10 mM NaCl, after stimulation by f22 DNA (100 fmol) or *DpnI*-cleaved plasmid DNA (13 ng, corresponding to 100 fmol of DNA fragments).

**p460 Requires Direct Contact with DNA to Assemble the Full DNA-PK Complex.** By screening DNA probes of different lengths for binding activity, we found that a DNA probe as short as 22 bp (f22) could still bind to Ku, as determined by the EMSA (Fig. 5A). In the absence of Ku, p460 was able to bind f22 and formed a complex that was retained in the well of the gel. When Ku was added, it formed a complex with DNA at the expense of the p460-dependent complex, and a Ku-p460 complex failed to form (compare Fig. 5A to Figs. 2A and 3A). Thus, Ku and p460 competed for binding to f22 DNA.

The f22 DNA strongly activated the p460 kinase, but the addition of Ku was capable of completely inhibiting this kinase activity (Fig. 5B). Thus, the p460 kinase activity could not possibly be due to contamination by Ku. By contrast, when *DpnI*-digested plasmid DNA was substituted for f22, so that larger DNA fragments were present, the addition of Ku stimulated the p460 kinase activity up to 5-fold. This result suggests that Ku was able to stimulate the p460 kinase only when the DNA fragments were large enough for Ku and p460 to each contact the DNA directly and form an active Ku-p460 complex.

## DISCUSSION

Previous studies required the presence of both Ku and p460 to activate the kinase (12), leading to the assumption that both subunits were required for kinase function. To examine this assumption critically, p460 was purified so that it was free of significant contamination by Ku (Figs. 1A and 2B). Surprisingly, purified p460 was capable of binding to DNA in the absence of Ku (Fig. 2A). Two distinct complexes formed between p460 and f32 DNA. One complex migrated with a mobility slightly faster than the complex of Ku-p460 and f32, and appears to represent a complex of p460 with f32. A second more abundant complex was retained in the well of the gel and remains undefined.

The DNA binding activity of p460 was strongest in low salt and decreased sharply when the salt concentration increased. Binding to the f32 DNA probe was competed effectively by single-stranded DNA in the form of poly(dT) and by supercoiled or linearized plasmid DNA. Thus, p460 binding to DNA was salt-labile and not specific for DNA ends, in marked contrast to Ku binding.

The binding of p460 to DNA was biochemically functional, because formation of a complex between p460 and DNA was

accompanied by activation of the kinase. Activation was specific for double-stranded DNA ends, because both supercoiled plasmid DNA and single-stranded DNA in the form of poly(dA) or poly(dT) failed to activate p460 kinase. Thus, although p460 bound to DNA without specificity for DNA ends, only double-stranded DNA ends activated the kinase.

Is it possible that p460 bound to DNA and showed kinase activity due to a small amount of Ku contamination in the preparation? Several different experiments ruled this out. (i) The level of contamination was measured both by immunoblot and by EMSA, allowing measurements of Ku in terms of both immunoreactivity and DNA binding activity. By immunoblot, the molar ratio of Ku to p460 was between 1:2,000 and 1:4,000. By EMSA, the amount of p460 binding activity was more than 100-fold too high to be explained by the most conservative estimate of the amount of Ku protein or the amount of Ku binding activity in the preparation (Fig. 2). (ii) The DNA binding properties of p460 and Ku were significantly different. Unlike Ku, p460 binding to f32 DNA was salt-labile and inhibited by both supercoiled DNA and single-stranded DNA. (iii) When the p460 preparation was tested in 150 mM or 200 mM NaCl buffer, kinase activity could not be detected unless purified Ku was added (Fig. 3B). (iv) Most significantly, both binding and activation of p460 with f22 were actually inhibited if Ku was added to the p460 preparation in a 10 mM NaCl buffer (Fig. 5). This striking result is explained by noting that Ku has a DNA footprint of 25–30 nucleotides (12, 16), suggesting that f22 was barely long enough to allow binding by either Ku or p460, but not both. Thus, these experiments rule out any confounding role for Ku contamination. Furthermore, they demonstrate that p460 is capable of acting as a self-contained kinase that is activated by binding directly to DNA.

If the kinase function is completely contained in p460, what then is the role of Ku? Although p460 bound to f32 in 10 mM NaCl, the binding activity and kinase activity disappeared at 100 mM and 200 mM NaCl (Fig. 3). The addition of Ku stabilized the binding of p460 to DNA and restored kinase activity at physiological salt concentrations. Also, Ku had a marked effect in stimulating the p460 kinase activity when linearized plasmid DNA was used for activation, but only a modest effect when the much smaller DNA from *DpnI*-cleaved plasmid was used (Fig. 4). Because the DNA binding of p460 is salt-labile and competed by supercoiled plasmid DNA, one role for Ku may be to stabilize the binding of p460 to DNA ends.

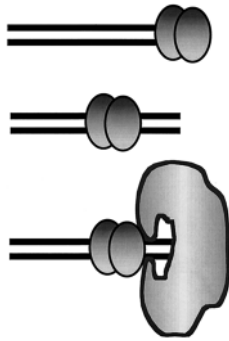


Fig. 6. Model for the assembly and activation of DNA-PK *in vivo*. Ku binds to the double-stranded DNA end, translocates in an ATP-independent manner to uncover the DNA end, and stabilizes the binding of p460 to the DNA end. Cooperative interactions among Ku, p460, and the DNA lead to efficient activation of the kinase. Our data indicate that both Ku and p460 contact the DNA, but their relative positions at the DNA end remain undetermined.

Gottlieb and Jackson (12) have reported that purified p460 did not have measurable kinase activity in the absence of Ku. There are several possible explanations for the difference between their results and ours. They used linearized plasmid DNA to activate the kinase, but we found this to be a much less efficient substrate for activation than shorter DNA fragments, probably because p460 binds well to both internal DNA sites and DNA ends. They used small amounts of either Sp1 or HSP70 protein as the phosphorylation substrate. By contrast, we used a peptide substrate optimized for DNA-PK at a 200-fold higher molar concentration. These differences in the length of the DNA and in both type and amount of the protein substrate may explain why p460 kinase activity was detected in our experiments, but not in those of Gottlieb and Jackson (12).

Yaneva *et al.* (28) published a report that claimed to observe DNA binding and activation of p460 kinase in the absence of Ku. However, there were significant differences between their results and ours. They did their experiments in 50 mM NaCl, a salt concentration that we found permits only weak activation of p460 in the absence of Ku. They reported that p460 binding and activation by an 18-bp DNA fragment were unaffected by the addition either Ku or supercoiled plasmid DNA, whereas we found that p460 binding and activation by f22 were strongly inhibited by Ku or supercoiled DNA. Significantly, they used two p460 preparations in which their estimates for molar Ku contamination (1:110 and 1:25) were 18- and 80-fold higher than ours (1:2,000). In our experiments, Ku contamination at a molar ratio of 1:100 produced significant levels of DNA binding (Fig. 2B) and kinase activation. Furthermore, the actual contamination in their experiments might have been even higher, because their estimate was based on the cross-linking of Ku to a chromophore 1 nucleotide from the end of a DNA fragment and the assumption that Ku would not be displaced from the DNA end by p460. Thus, the properties reported by Yaneva *et al.* (28) for p460 are indistinguishable from the properties of Ku-p460 and may be explained by Ku contamination.

A model for how DNA-PK assembles on a DNA end is summarized in Fig. 6. Ku binds to the DNA end and translocates to an internal position (17), uncovering the DNA end and forming a more stable complex with the DNA (29, 30). The uncovered DNA end is then bound by p460 to form a DNA-PK complex in which cooperative interactions among p460, Ku, and the DNA lead to efficient activation of the kinase. This model potentially explains why DNA nicks, hairpin ends (21), and cisplatin-damaged DNA (21) fail to activate DNA-PK. These DNA substrates are bound by Ku, but the resulting Ku-DNA complex may not expose the appropriate DNA structure required for activation of the p460 kinase.

In conclusion, these experiments clarify the interaction of DNA with p460 that leads to activation of the kinase. Although the ability of p460 to be activated on its own may not be relevant at physiological salt *in vivo*, our results show that the p460 molecule must contain a functional kinase domain and a DNA-binding domain. They also raise the possibility that p460 may have partners other than Ku to guide it to DNA and, conversely, that Ku may guide kinases other than p460 to DNA ends.

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