

GRANT NUMBER DAMD17-94-J-4350

TITLE: Genes Involved in DNA Double-Strand Break Repair:
Implications for Breast Cancer

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REPORT DATE: October 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 4

19990811 097

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY <i>(Leave blank)</i>	2. REPORT DATE October 1998	3. REPORT TYPE AND DATES COVERED Final (1 Oct 94 - 30 Sep 98)	
4. TITLE AND SUBTITLE Genes Involved in DNA Double-Strand Break Repair: Implications for Breast Cancer		5. FUNDING NUMBERS DAMD17-94-J-4350	
6. AUTHOR(S) Gilbert Chu, M.D., Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Stanford University Stanford, California 94305		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012		10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT <i>(Maximum 200)</i> Breast tissue is particularly sensitive to the carcinogenic effects of ionizing radiation, which creates DNA double-strand breaks. These double-strand breaks are repaired by an end-joining mechanism that is also utilized in V(D)J recombination to generate immunological diversity. We and others have shown that end-joining requires DNA-dependent protein kinase (DNA-PK), which consists of a regulatory subunit, Ku, and a catalytic subunit, p460. We found that intact DNA-PK is not required for accumulation of p53 or cell cycle arrest following DNA damage. Instead, DNA-PK appears to play a direct role in DNA repair. The structure of p460 was determined by electron crystallography, and found to include a potential DNA binding site, consistent with our discovery that p460 can be activated by a direct interaction with DNA in the absence of Ku. Two DNA intermediates during V(D)J recombination, hairpin ends and nicked DNA, fail to activate DNA-PK, imposing constraints on models for how p460 interacts with DNA. Finally, p460 appears to recruit additional factors to DNA ends, thus inducing assembly of a holoenzyme in preparation for DNA end-joining. Future experiments will be directed towards a full understanding of this critical pathway for suppressing breast cancer.			
14. SUBJECT TERMS Breast Cancer		15. NUMBER OF PAGES 19	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

FOREWORD

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INTRODUCTION

Significance of double-strand breaks

DNA double-strand breaks (DSBs) may be the most disruptive form of DNA damage. If left unrepaired, they lead to broken chromosomes and cell death. If repaired improperly, they can lead to chromosome translocations and cancer. Humans are at risk for DSBs from exogenous agents. The paradigm agent, ionizing radiation (IR), is present in the environment from the decay of radon and radon daughters, which accumulate to high levels in homes built on land reclaimed from mining, leading to increased cancer risk. Ionizing radiation can cause cancers in many tissues, with breast tissue being particularly sensitive (6, 12). Mice exposed to IR show a dose-dependent increase in breast cancer (59). Women exposed by the atomic bombs detonated over Hiroshima and Nagasaki (42) or treated with radiation for post partum mastitis (51) had increased risks for breast cancer.

DSBs are also created during the treatment of cancer by the use of IR or anticancer drugs, such as bleomycin, which produces oxidative free radicals, or etoposide and adriamycin, which inhibit topoisomerase II to create protein-bridged DSBs. Humans are also at risk for DSBs from endogenous agents. Oxidative metabolism generates free radicals and subsequent strand breaks. V(D)J recombination generates DSBs during rearrangement of genes encoding B cell immunoglobulins and T cell receptors (53).

In response to the threat of DSBs, cells have evolved at least two independent pathways for repairing DSBs, by homologous recombination or by nonhomologous DNA end-joining. We have chosen to focus on understanding the dominant mechanism in mammalian cells, nonhomologous end-joining, which has proven to be involved in both resistance to IR and V(D)J recombination.

Mutant cell lines

Immunological diversity is generated by V(D)J recombination, a site-specific cleavage of the chromosome followed by an end-joining reaction to bring the free DNA ends together (53). The possibility that DSB repair and V(D)J recombination might share the same biochemical pathway was first recognized in the severe combined immunodeficiency (scid) mouse. The scid mouse lacks mature B and T cells due to a defect in V(D)J recombination (31) and is also hypersensitive to IR due to a defect in DSB repair (2, 15, 22). Screening of IR sensitive cell lines led to the identification of four genetic complementation groups with defects in both DSB repair and V(D)J recombination (38, 56). Since efforts were underway to complement these rodent cell lines with human genes, the genes for the complementation groups were designated *XRCC*, for X-ray cross-complementing. *XRCC7* mutant cells (scid mouse cells and V3 hamster cells) were severely defective in coding joint formation but only mildly defective in signal joint formation. By contrast, *XRCC4*, *XRCC5*, and *XRCC6* mutant cells were severely defective in both coding and signal joint formation.

Ku and DNA-dependent protein kinase

Ku was originally identified as a major autoantigen in scleroderma-polymyositis overlap syndrome (34, 47). Ku is a heterodimer of 70 and 86 kDa (Ku70 and Ku86) that binds with strong affinity to DNA ends, stem-loop structures, or transitions between double-stranded DNA and two single strands (13, 35). Once Ku binds to DNA, it can translocate along the DNA in an ATP-independent manner, so that three or more Ku molecules can bind to a single linear DNA fragment (10, 37). Ku is also a DNA-dependent ATPase that is activated by both double and single-stranded DNA (7), and motifs for potential ATP binding sites are found in both Ku70 and Ku86. Ku has also been reported to have an ATP-dependent 3' to 5' helicase activity (58).

Ku is the regulatory subunit for DNA-dependent protein kinase (DNA-PK), which has the unusual property of being activated by DNA ends (19). DNA-PK contains an enormous catalytic subunit of 460 kDa (p460) that is activated when Ku binds to DNA. Interestingly, DNA-PK will confer ATPase activity to dephosphorylated Ku protein (7). However, attempts to identify *in vivo* substrates have been inconclusive.

Genes for double-strand break repair

In searching for proteins that recognized altered DNA structures, we identified a DNA end-binding (DEB) activity in cell extracts, which bound specifically to DNA ends (44). A large number of IR-sensitive cell lines were screened, and DEB factor was absent in three different cell lines, all belonging to the complementation group for *XRCC5* (44). DEB factor proved to be both biochemically and antigenically similar to Ku (16, 45). To see if *XRCC5* cells are defective in Ku, we transfected expression vectors for Ku70 and Ku86 into the mutant *XRCC5* hamster cells. Transfection of human Ku86 rescued the mutant hamster cells for DEB activity, DNA-PK enzymatic activity, IR resistance, and V(D)J recombination (54, 55). These results demonstrated that Ku is involved in the repair of DNA DSBs produced by ionizing radiation or V(D)J recombination.

The discovery that Ku was defective in *XRCC5* mutant cells raised the possibility that p460 might be defective in *XRCC7* cells (4, 24, 39). Indeed, DNA-PK enzymatic activity and p460 protein levels were severely reduced in both *scid* and *V3* cells. Transfection of *V3* and *scid* cells with genomic *yACs* carrying the p460 gene restored DNA-PK enzymatic activity, IR-resistance, and coding joint formation. Furthermore, the *p460* gene is mutated in *scid* cells, producing a premature termination codon in the putative kinase domain that truncates the C-terminal 83 amino acids (5, 9). As in *Ku86* knockout thymocytes, *scid* thymocytes accumulate hairpin coding ends (61). Therefore, Ku and DNA-PK are required for the proper processing of hairpin ends. Ku or DNA-PK could have an endonuclease activity that opens the hairpin ends directly, but such an endonuclease activity has not been reported. More likely, DNA-PK enzymatic activity makes the hairpin accessible to a still unidentified hairpin endonuclease.

The only known *XRCC4* cell line, XR-1, is rescued by transfection of a cDNA encoding a 37 kDa protein, restoring both IR-resistance and V(D)J recombination to wild type levels (29). *XRCC4* is a novel gene, lacking homology to any known gene in the current data base. It is deleted in XR-1 cells, and therefore not essential for growth. The sensitivity of XR-1 cells increases dramatically in the G1 phase of the cell cycle (17), and the proposed role for *XRCC4* in stimulating ligase activity (20) must account for this phenomenon.

DNA end-joining in intact cells

In vivo properties of the end-joining reaction have been revealed by experiments with intact mammalian cells. When naked DNA was transfected into mammalian cells, end-joining either occurred by direct joining of the ends or by involving deletions back to regions of microhomology of 1 to 6 bases (49, 50). The latter joining events appeared to be directed by base pairing of the microhomology regions, thus aligning the DNA ends for subsequent steps in the end-joining reaction.

To study end-joining of chromosomal DNA, restriction enzymes have been used to introduce DSBs at specific chromosomal sites. Restriction enzymes were electroporated into Chinese hamster ovary cells, which are hemizygous at the *APRT* (adenine phosphoribosyl transferase) locus (41). Colonies of viable cells containing mutations in *APRT* were then analyzed at the DNA sequence level. Restriction enzyme-induced mutations consisted of insertions, small deletions up to 36 bp, and combinations of insertions and deletions at the cleavage sites. Most of the deletions involved the utilization of microhomology of 1 to 4 bases at

the recombination junctions. Thus, the end-joining reactions observed with transfected naked DNA and chromosomal DNA shared key characteristics: nucleotide insertions and nucleotide deletions directed by microhomology. Furthermore, when chromosomal DNA was cleaved by a rare cutting restriction enzyme, the subsequent end-joining required Ku (30).

V(D)J recombination junctions provide additional insight into end-joining of broken chromosomes (53). Coding joint formation occurs with both insertion and deletion of nucleotides. Most insertions occur by mechanisms specific for lymphoid cells. Palindromic (P-nucleotide) insertions are generated by the asymmetric cleavage of the hairpin coding ends created by RAG1/RAG2 cleavage. N-nucleotide insertions are generated by the random addition of nucleotides catalyzed by lymphoid specific terminal deoxynucleotidyl transferase (TdT). Nucleotide deletion is also best studied in TdT knockout mice, since a high frequency of TdT-catalyzed insertions can obscure the nature of the deletions. In the absence of TdT, 75% of the joining events contain deletions directed by microhomology regions of 1 to 5 nucleotides (18, 25). Thus, the joining reaction for coding ends during V(D)J recombination confirms the characteristics observed for random DSBs in nonlymphoid cells. In contrast to coding joint formation, signal joint formation is precise and dependent on Ku, but not on an intact kinase domain in p460. Thus, the mechanisms that lead to the insertion and deletion of nucleotides are somehow suppressed during the formation of signal ends.

DNA end-joining in cell-free extracts

Progress towards defining nonhomologous end-joining in a cell-free system has been slow compared to progress in identifying the relevant genes. End joining was studied in *Xenopus* extracts by recircularization of linear DNA molecules (40, 57). In contrast to intact mammalian cells, *Xenopus* extracts did not produce nucleotide deletion beyond losses from protruding single strands. End-joining in mammalian extracts has been studied by the recircularization of plasmid DNA cleaved with restriction enzymes. Almost all events involved direct joining of the cohesive ends, but there were a small number of misrejoined molecules with insertions or deletions utilizing regions of microhomology. It was possible to fractionate extracts into four different end-joining activities (23). The activity that utilized microhomology near the ends could be purified extensively (32). However, none of the activities was dependent on the presence of Ku protein. Therefore, these cell free systems failed to detect the Ku-dependent end-joining mechanism in mammalian cells.

There has been success in obtaining DNA end-joining in cell-free extracts for the special case of V(D)J recombination (8, 43). Cleavage by RAG1 and RAG2 was followed by a rejoining reaction with the appropriate properties: precise joining of the signal ends and utilization of microhomology for the coding ends, albeit with low efficiency. For the special case of signal end joining, the reaction appeared to be specific for Ku since it was inhibited by anti-Ku antibodies (8).

Purpose and scope of the research

In summary, at the time this proposal was written, we and others had found evidence suggesting that Ku and DNA-PK were involved in DSB repair *in vivo*. Because of the limited progress in recovering DSB repair *in vitro*, the roles of Ku, DNA-PK, and XRCC4 remained poorly defined. Furthermore, additional proteins must be required for the reaction, and these proteins and their genes remain unidentified. Our research for this grant addressed these issues as follows.

1. Demonstration that Ku86 defines the genetic defect and restores X-ray resistance and V(D)J recombination to complementation group 5 hamster cells.

2. Demonstration that intact DNA-PK is required for accumulation of p53 or cell cycle arrest following DNA damage.
3. Demonstration that DNA can bind and activate p460 in the absence of Ku.
4. Determination of the structure of p460 by electron crystallography.
5. Demonstration that hairpin ends and nicked DNA fail to activate DNA-PK.
6. Demonstration that additional factors assemble with Ku and p460 at DNA ends.

BODY

Ku86 defines the genetic defect and restores X-ray resistance and V(D)J recombination to complementation group 5 hamster cells

We showed that the *XRCC5* cell lines XR-V9B and XR-V15B contained mutations in the *Ku86* gene (11), leading to internal amino acid deletions in Ku86. Other *XRCC5* cell lines were subsequently reported to contain severely truncated proteins (52). We further showed that transfection of an expression vector for Ku86 fully restored both resistance to ionizing radiation and V(D)J recombination to the *XRCC5* mutant cell line XR-V15B. Table 1 summarizes the genetic defects in mutant cell lines for the proteins known to be involved in DSB repair.

As expected, the generation of *Ku86* knockout mice produced a severe combined immunodeficiency due to an absence of both T and B cells (36, 61), as seen for scid mice. Both coding and signal joint formation were impaired, and the coding joint defect was accompanied by accumulation of hairpin coding ends in *Ku86* knockout thymocytes.

Table 1. Proteins involved in double-strand break repair. Genetic complementation groups, corresponding to the X-ray cross complementing genes, *XRCC4* - *XRCC7*, have been assigned for cell lines defective in double-strand break repair and V(D) recombination. All cell lines are hypersensitive to IR. The *XRCC6* mutant cell line was generated by targeted knockout of mouse embryonic stem cells.

Mutated gene (protein)	V(D)J defect	Mutant cells	Mutation
<i>XRCC4</i>	coding/signal	XR-1	gene deletion (29)
<i>XRCC5</i> (Ku86)	coding/signal	XR-V9B	internal a.a. deletion (11)
		XR-V15B	internal a.a. deletion (11)
		xrs4	truncated 287 a.a. protein (52)
		xrs5	?
		xrs6	truncated 24 a.a. protein (52)
<i>XRCC6</i> (Ku70)	coding/signal	ES cells	knockout (21)
<i>XRCC7</i> (p460)	coding	scid	terminal 83 a.a. truncation (5, 9)
		V3	?

Intact DNA-PK is not required for accumulation of p53 or cell cycle arrest following DNA damage

In response to DNA damage, cells transduce a signal that leads to accumulation and activation of p53 protein, transcriptional induction of several genes, including *p21*, *gadd45*, and *gadd153*, and cell cycle arrest. One hypothesis is that the signal is mediated by DNA dependent protein kinase (DNA-PK), which consists of a catalytic subunit (p460) and a regulatory subunit (Ku). DNA-PK has several characteristics that support this hypothesis: DNA-PK is activated by DNA ends, DNA-PK will phosphorylate p53 and other cell cycle regulatory proteins *in vitro*, and p460 shares homology with *ATM*, which is mutated in ataxia telangiectasia and involved in signaling the p53 response to ionizing radiation. The hypothesis was tested by analyzing early passage fibroblasts from scid mice, which are deficient in DNA-PK. After exposure to ionizing radiation, ultraviolet radiation, or methyl methanesulfonate, scid and wild type cells were indistinguishable in their response (46). The accumulation of p53, induction of *p21*, *gadd45*, and *gadd153*, and arrest of the cell cycle in G₁ and G₂ occurred normally. Therefore, intact DNA-PK is not required for the p53 response or cell cycle arrest after DNA damage.

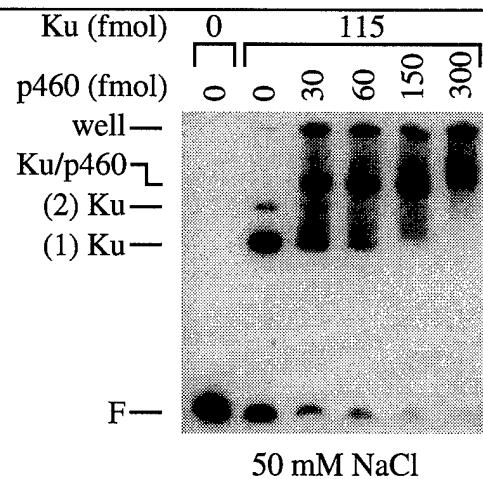
EMSA for assembly of Ku and p460 onto a DNA end

The work described in Background and Significance identified four genes involved in a common biochemical pathway for rejoining free DNA ends shared by DSB repair and V(D)J recombination. To study this pathway biochemically, we previously developed an electrophoretic mobility shift assay (EMSA) for measuring Ku binding to DNA ends. This assay was extremely sensitive for Ku but failed to reveal the assembly of the full DNA-PK enzymatic complex on DNA ends. We therefore developed a new EMSA that would circumvent this problem. In the new EMSA, extracts were incubated with a short DNA probe of only 32 bp (f32). We discovered that the short DNA probe was not affected by nonspecific DNA binding proteins, so that supercoiled competitor DNA could be omitted from the binding reaction.

In the new EMSA, incubation of purified Ku with f32 probe DNA produced a major protein-DNA complex, corresponding to a single binding event of Ku to f32, and a minor complex, corresponding to two binding events of Ku to f32 (Fig. 1). These complexes were also observed in the old EMSA. Incubation of purified Ku and purified p460 with f32 produced two additional lower mobility complexes (Fig. 1). One complex corresponded to the binding of one Ku and one p460 molecule. A second complex was retained in the well of the gel and therefore corresponded to the binding of at least two Ku/p460 molecules. Although this complex may be a nonspecific aggregate, we plan to explore the possibility that it represents a well defined synopsis of two DNA molecules mediated by the association of two p460 molecules.

Incubation of f32 with crude HeLa extracts produced a series of complexes that comigrated with the complexes seen in Fig. 1. The identity of the complexes from the HeLa extracts was confirmed in several ways. First, three different monoclonal antibodies against different epitopes of p460 were added to the binding reaction and were found to either supershift or disrupt the complex that comigrated with the Ku/p460/DNA complex (V. Smider and G. Chu, manuscript in preparation). Second, when antibodies against Ku were added to the binding reaction, they supershifted the complexes that comigrated with the Ku/DNA and Ku/p460/DNA complexes. Third, when the f32 probe was incubated with extracts from V3 and scid cells, which are defective in p460 (see Table 1), the low mobility Ku/p460/DNA complex was specifically absent. Therefore the new EMSA provided a rapid assay for purifying Ku and p460 from crude extracts.

Figure 1. EMSA for assembly of DNA-PK on DNA. The ^{32}P -labeled f32 DNA probe was incubated with purified Ku plus different amounts of purified p460. After 10 min. at room temperature, the products were resolved by electrophoresis in nondenaturing polyacrylamide gel and then visualized by autoradiography. The position of the free DNA probe (F) is shown at the bottom of the gel. With Ku alone, two complexes were observed, corresponding to the binding of 1 and 2 Ku molecules to the DNA. With Ku and p460, new complexes were observed, one corresponding to the binding of 1 Ku and 1 p460 molecule to the DNA (Ku/p460) and one super-large complex that was retained in the well of the gel.



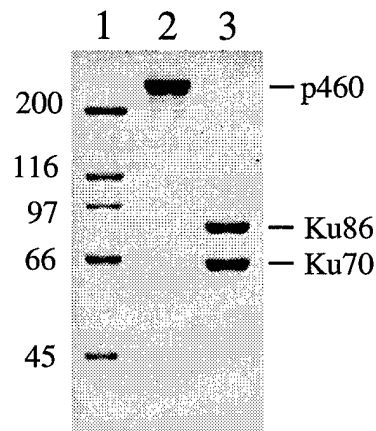
Purification of Ku and p460

To further study the biochemical roles of Ku and p460 in double-strand break repair, it was critically important to obtain highly purified preparations of both proteins. During the course of purification, we noted the presence of a complex that appeared to contain p460 in the absence of the specific Ku/DNA complex. We therefore developed a protocol to successfully purify Ku and p460 separately (Fig. 2).

In particular, the p460 preparation was freed of contaminating Ku by the use of an oligonucleotide affinity column consisting of 1 ml of NHS-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 (26). The oligonucleotide column was then eluted with a 0.1 to 0.5 M NaCl gradient. Under these conditions, p460 eluted at 0.25-0.30 M NaCl, leaving the majority of Ku bound to the resin, since Ku 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 flowthrough while the contaminating Ku and a minor fraction of p460 remained bound to the resin. The level of Ku contamination was measured both by immunoblot and EMSA. The molar ratio of Ku to p460 was between 1:2000 and 1:4000 by the use of serial dilutions.

Figure 2. SDS-PAGE of separately purified Ku and p460.

Ku and p460 were separately purified to homogeneity from human placenta by chromatographic methods. The preparations were then analyzed by SDS-PAGE followed by staining with Coomassie blue. The p460 preparation (lane 2) was freed of Ku contamination down to a molar ratio between 1:2000 and 1:4000 by using an oligonucleotide affinity column. The Ku preparation (lane 3) contained no detectable p460.



DNA can bind and activate p460 in the absence of Ku

Previous reports suggested that Ku was required for both DNA binding and kinase activity (19). To our surprise, we discovered that in low salt buffer (10 mM NaCl), the highly purified p460 bound to f32 DNA in the EMSA, even in the absence of Ku. Two distinct p460/DNA complexes were observed: one that migrated slightly faster than the Ku/p460/DNA complex, and a second that was retained in the well of the gel, corresponding to a complex containing at least two p460 molecules (Fig. 6, lanes 1 and 2). Furthermore, the binding was biochemically significant since the purified p460 was activated by the addition of DNA to phosphorylate its specific peptide substrate (27).

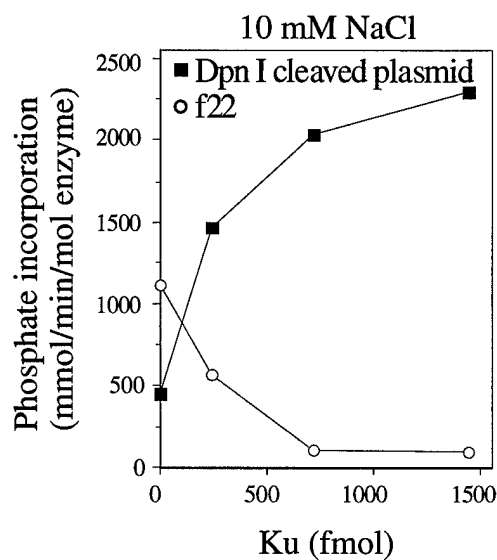
Is it possible that these results were caused by a small amount of Ku contamination in the preparation? Several different experiments ruled this out. First, the level of Ku contamination was extremely low, less than 1 part in 2000. We calculated that the level of p460 DNA binding activity was 100-fold too high to be explained by the most conservative estimate of the amount of Ku in the preparation. Second, the DNA binding properties of p460 and Ku were significantly different. Unlike Ku, p460 binding to f32 DNA was salt labile and competitively inhibited by both supercoiled DNA and single-stranded DNA. Third, when the p460 preparation was tested in 150 mM or 200 mM NaCl buffer, no kinase activity was observed unless purified Ku was added. Fourth, and most significantly, p460 was capable of binding to a 22 bp DNA fragment (f22), which was too short to allow the simultaneous binding of both Ku and p460 (data not shown). Since Ku has a DNA footprint of 25-30 nucleotides (19, 35), the f22 DNA was not long enough to allow binding by both Ku or p460. In a 10 mM NaCl buffer, p460 kinase activity was strongly activated by the addition of f22 (Fig. 3). Subsequent addition of Ku actually inhibited the kinase activity of p460, demonstrating conclusively that the activation of p460 kinase does not require Ku. Together, 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. The addition of Ku stabilized DNA binding and restored kinase activity in the higher salt concentrations. The addition of Ku had a marked affect in stimulating the p460 kinase activity when linearized plasmid DNA or Dpn I cleaved plasmid was used for activation (Fig. 3). Since the DNA binding of p460 is salt labile and competed by supercoiled plasmid DNA, the role for Ku may be to stabilize the binding of p460 to DNA ends. Although Ku is not absolutely required for the activation of the p460 kinase function by DNA ends, Ku directs p460 more efficiently to the DNA ends, particularly at physiological salt concentrations.

Gottlieb and Jackson (19) previously 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 internal DNA sites as well as to 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 concentrations 200-fold higher on a molar basis. These differences in the length of the DNA substrate and both type and amount of the phosphorylation substrate may explain why Gottlieb and Jackson failed to detect the p460 kinase activity.

Figure 3. The p460 kinase is activated by DNA in the absence of Ku.

Kinase activity was measured by incubating protein with ^{32}P - γ -labeled ATP and a peptide containing a sequence specific for DNA-PK, EPPLSQEAFADLWKK (27). Peptide phosphorylation was completely dependent on addition of DNA ends. Kinase activity was measured for 70 fmol of p460 with the different amounts of Ku in buffer containing 10 mM NaCl. The p460 kinase was strongly activated by DNA, even in the absence of Ku. For Dpn I cleaved plasmid DNA, the addition of Ku stimulated the activation of p460. However, for f22 DNA, which was too short to allow the simultaneous binding of both Ku and p460 (data not shown), the addition of Ku resulted in the inhibition of kinase activity. This demonstrated conclusively that p460 kinase activation does not require Ku.

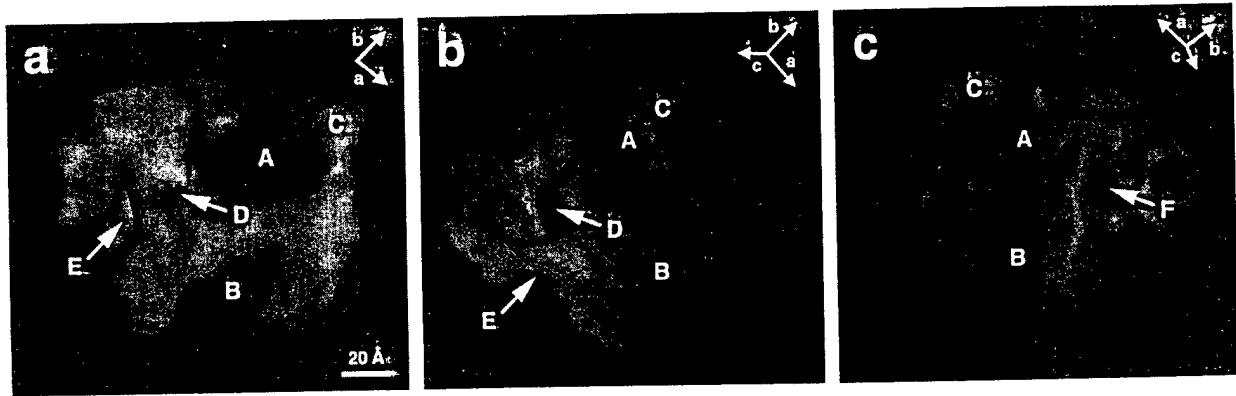
**Structure of purified p460 by 2-dimensional crystallography**

In collaboration with Drs. Kerstin Leuther and Roger Kornberg at Stanford, we succeeded in obtaining 2-dimensional crystals of purified p460 imbedded in a lipid monolayer. This technique has been used in conjunction with electron beam microscopy to obtain the structure of other very large proteins, including RNA polymerase II both with and without other transcription factors to a resolution of 19 Angstroms (28). The advantage of the technique is that a crystal structure could be obtained with as little as 100 μg of purified p460.

We have now obtained a full 3-dimensional reconstruction of the p460 to a resolution of 20 Angstroms (Fig. 4). The structure contains an open channel, similar to those seen in other double-stranded DNA binding proteins. The structure also contains an enclosed cavity with three openings, one adjacent to the open channel. Each opening is large enough to accommodate single-stranded DNA.

To gain insight into how the DNA interactions of p460 might reflect its structural features, we determined kinase activation as a function of DNA length. Efficient activation of the kinase required DNA at least 15 bp in length, consistent with dimensions of the open channel. Because the enclosed cavity in p460 might represent a single-stranded DNA binding site, we investigated the effect of single-stranded DNA on the kinase. Activation of the kinase was inhibited by single-stranded DNA with both competitive and noncompetitive kinetics, consistent with distinct but interacting binding sites for single-stranded and double-stranded DNA, as suggested by the structure.

Figure 4. Three-dimensional structure of the p460 molecule. **a.** View perpendicular to the lipid layer. There are two open channels, A and B, on opposite sides of the molecule. The feature labeled C indicates protein density at the end of a potentially flexible arm surrounding channel A. **b.** View showing the opening D of the enclosed cavity as it emerges adjacent to channel A. **c.** View showing the other side of the molecule. Arrow F indicates a third opening of the enclosed cavity.



Hairpin ends will bind Ku but fail to activate DNA-PK

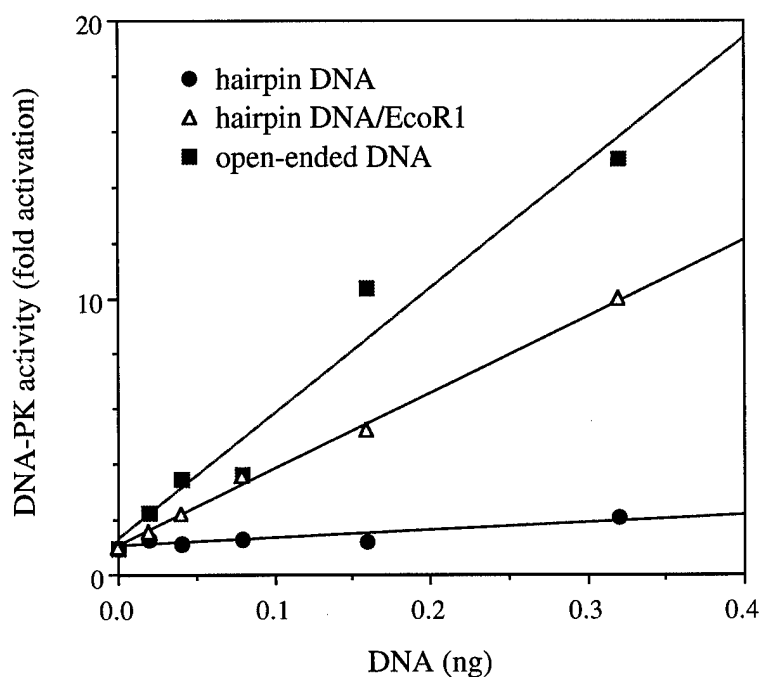
The lymphoid specific proteins RAG1 and RAG2 initiate V(D)J recombination by recognizing recombination signal sequences adjacent to V, D, or J coding sequences and catalyzing a coordinated cleavage at two sites, each at the border between a signal sequence and a coding sequence (33, 60). Cleavage occurs in a two step reaction, first nicking the DNA to leave a 3'-hydroxyl and 5'-phosphate, and then mediating a nucleophilic attack of the 3'-hydroxyl on the opposite strand to form a covalently closed hairpin coding end and a blunt signal end.

Following cleavage, joining of the DNA ends requires DNA-dependent protein kinase (DNA-PK). Ku, the regulatory subunit, binds to DNA ends to direct the activation of p460, the catalytic subunit. *In vivo*, the mouse scid mutation in p460 disrupts joining of the hairpin coding ends but preserves significant joining of the open signal ends (31).

To better understand the role of DNA-PK in V(D)J recombination, a DNA molecule with perfect hairpin ends was synthesized from 3 oligonucleotides in a collaboration with Dr. Susanna Lewis, at University of Toronto. Two of the oligos contained palindromic sequences to allow the formation of the hairpin ends, and the third oligo annealed to the overhanging ends of the other 2 oligos to form a duplex DNA molecule. This hairpin-ended DNA molecule was then tested for its interactions with DNA-PK. Surprisingly, hairpin-ended DNA was an extremely poor substrate for activating DNA-PK, producing at most 7% of the kinase activity observed for open-ended DNA of approximately the same length. Although Ku binding to hairpin ended DNA was 2 to 4-fold less efficient than to open-ended DNA, this modest decrease in affinity does not appear to fully account for the failure of hairpin ends to activate the kinase. Thus, p460 may be recruited to hairpin ends in a nonfunctional form, or p460 may discriminate between hairpin and open ends.

Figure 5. Failure of hairpin ends to activate DNA-PK.

DNA-PK was incubated with a 68 bp hairpin-ended DNA molecule (closed circles), the hairpin-ended DNA cleaved with Eco RI (open triangles), or a 70 bp open-ended DNA molecule (closed squares) in the presence of peptide substrate. DNA-PK activity was measured as the fold increase in phosphorylation of the peptide in the presence of DNA over phosphorylation in the absence of DNA. The hairpin-ended DNA failed to activate DNA-PK. By contrast, DNA-PK was strongly activated by either the Eco RI cleavage products or the open-ended DNA.



We were surprised by the failure of hairpin-ended DNA to fully activate DNA-PK. Therefore, we decided to examine the effect of DNA nicks on DNA-PK, since nicks bind to Ku (3) and are created as a DNA intermediate during V(D)J recombination (33). Thus, p460 might be recruited and activated at the nicking step, so that when RAG1 and RAG2 complete the cleavage reaction, DNA-PK could assemble on the hairpin end in its activated form. We therefore tested the activation of DNA-PK with two different nicked DNA substrates: the 68 bp hairpin molecule containing a single nick, and plasmid DNA nicked with DNase I. As observed with hairpin-ended DNA, both nicked substrates failed to activate DNA-PK, yielding no more than 7% of the activity observed with open-ended DNA.

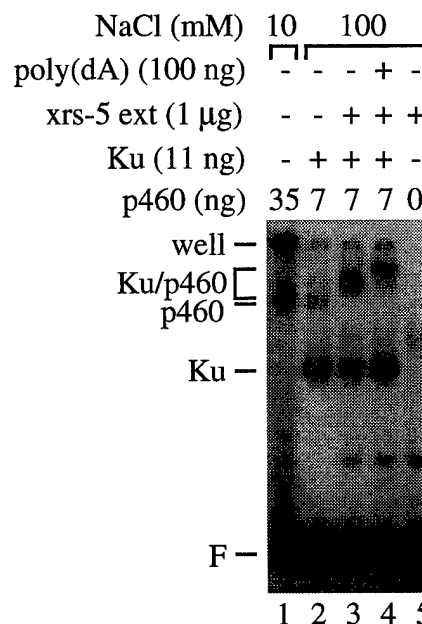
What are the implications of these results for V(D)J recombination? DNA-PK is required for efficient hairpin processing (48, 61) but fails to be activated by hairpin ends. In addition, the kinase activity of p460 need not be intact for signal ends to be joined as seen in scid cells (4, 38), even though it is fully activated by blunt signal ends. This apparent paradox can be explained if processing of hairpin ends requires activation of DNA-PK assembled on the co-generated signal end.

We propose a model for V(D)J recombination in which DNA-PK participates in processing hairpin ends by autophosphorylating Ku and p460 in trans. In particular, Ku bound to hairpin ends would be transphosphorylated by DNA-PK bound to the co-generated signal ends. Ku has an ATPase activity that is activated by DNA-PK phosphorylation (7) and has been reported to have a helicase activity as well (58). Once activated by DNA-PK, the Ku ATPase might unwind the hairpin into a looped structure to facilitate cleavage by a putative hairpin endonuclease leading to subsequent joining of the coding ends. In this model, the signal ends are joined directly without processing, and so do not require Ku transphosphorylation. Ku bound to the signal ends might even be protected from phosphorylation by RAG1 and RAG2, which remain bound to the signal ends after cleavage *in vitro* (1). This explains the puzzling observation that signal joint formation is spared in scid mice, since Ku bound to the signal end would not be phosphorylated by p460 even in wild type mice.

Additional factors involved in assembly of a repair complex on DNA ends

When crude extracts from Ku deficient cells were added to Ku and p460 and then analyzed for protein-DNA complexes with f32 in the EMSA, we discovered the formation of a new complex with a slightly lower mobility and a far greater stability than the DNA-PK complex (see Fig. 6). The stabilization was due to at least two factors in the extract, since fractionation of the extract on an S-Sepharose column followed by a hydroxyapatite column produced two distinct peaks of activity. This result suggests that the formation of the DNA-PK complex on DNA ends serves as a platform for recruiting additional factors that enhance the activities of DNA-PK in end joining or participate in later steps of the end-joining reaction.

Figure 6 Crude extracts contain a factor that stabilizes the assembly of DNA-PK on DNA. The DNA probe f32 was incubated with various protein preparations or with poly(dA) as shown. The shifted mobility in lane 1 marks the position of the p460/DNA complex, which is only stable in low salt buffer (10 mM NaCl). In 100 mM NaCl, Ku and p460 produces complexes corresponding to the binding of Ku or Ku/p460 to DNA (lane 2). Addition of crude extract from Ku deficient *xrs-5* cells causes the Ku/p460 complex to shift upward and become much more intense, suggesting the recruitment of an additional protein to the complex and stabilization of the interaction of Ku/p460 with DNA (lane 3). By contrast, this complex is absent when the *xrs-5* extract was added in the absence of Ku and p460 (lane 5). The addition of poly(dA) leads to a further shift upward of the complex (lane 4), demonstrating that this complex now contains a second DNA binding site that, unlike the Ku DNA binding site, will recognize single-stranded DNA.

**CONCLUSIONS**

These experiments have dissected early steps in the pathway for DNA double-strand break repair. Since double-strand breaks are the most important lesions produced by ionizing radiation, and since exposure to ionizing radiation is a critical risk factor for breast cancer, these results will lead to progress in the prevention and treatment of breast cancer.

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