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Involvement of the Ku autoantigen in the cellular response to DNA double-strand breaks

[x-ray sensitivity/DNA repair/V(D)J recombination]

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ABSTRACT **The Ku autoantigen is a well-characterized heterodimer of 70 and 86 kDa that binds to DNA ends, but its cellular function has been obscure. An electrophoretic mobility-shift assay and Ku antisera were used to show that Ku or a closely related protein was deficient in three mutant hamster cell lines from x-ray-sensitive complementation group 5, which is characterized by defects in DNA double-strand break repair and V(D)J recombination. Furthermore, Ku protein expression was restored when the cells reverted to x-ray resistance. The Ku p86 gene maps to human chromosome 2q33–35, and group 5 cells are rescued by almost precisely the same region, 2q34–36. Thus, biochemical and genetic evidence suggests that Ku is involved in pathways for DNA recombination and repair. By its association with a DNA-dependent protein kinase activated by DNA ends, Ku may also initiate a signaling pathway induced by DNA damage, perhaps for cell cycle arrest.**

Despite extensive characterization, the cellular function of the Ku protein has remained enigmatic. Ku was first identified as an autoantigen in a patient with polymyositis–scleroderma overlap syndrome (1). It was subsequently found to be an autoantigen in patients with several other autoimmune diseases, including systemic lupus erythematosis, Graves disease, and Sjögren syndrome (2). Ku is a heterodimer of 70 kDa and 86 kDa polypeptides (p70 and p86); cDNAs for both subunits have been cloned (3–5). Ku protein binds to double-stranded DNA ends with 5′- or 3′-protruding ends or blunt ends, nicked DNA (6), and duplex DNA ending in stem–loop structures, apparently by recognizing transitions from double- to single-stranded DNA (7). Ku also associates with a 350-kDa polypeptide to form a DNA-dependent protein kinase (DNA-PK) that has the interesting feature of being activated by DNA ends (8). *In vitro* substrates of DNA-PK include p53, c-Myc, RNA polymerase II, Sp1, Oct-1, Oct-2, simian virus 40 T antigen, and Ku itself (9–11).

At least three genetic complementation groups of x-ray-sensitive rodent cells are defective in two pathways for resolving DNA ends: the repair of x-ray-induced DNA double-strand breaks (12–16) and V(D)J recombination of the genes encoding immunoglobulins and T-cell receptors (17, 18). Using an electrophoretic mobility-shift assay, we (19) recently identified a DNA end-binding (DEB) factor that was deficient in three independent mutant CHO cell lines (xrs5, XR-V15B, and XR-V9B), suggesting that DEB factor is involved in both double-strand break repair and V(D)J recombination. XR-V9B was originally assigned to x-ray complementation group 10 (14) but has now been reassigned (M. Zdzienicka, personal communication), so that xrs5, XR-V15B, and XR-V9B all belong to group 5 (20). Thus, group 5 appears to be characterized by a defect in DEB factor.

This paper presents evidence that Ku and DEB factor are closely related if not identical. Indeed, the data suggest that group 5 cells are defective for the Ku p86 gene.

MATERIALS AND METHODS

Cell Lines. The wild-type primary human fibroblast line IMR-90; wild-type CHO cell lines AA8, V79, and V79B; and x-ray-sensitive mutant CHO cell lines xrs5 (12), XR-V15B (13), and XR-V9B (14) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1 mM glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml).

Electrophoretic Mobility-Shift Assay for DEB Activity. Nuclear extracts were prepared by lysis of cells in 1% (vol/vol) Nonidet P-40 and extraction of the nuclei in high salt (19, 21). The extracts were tested for DEB activity by an electrophoretic mobility-shift assay (19). Briefly, 0.6 μg of nuclear extract protein was incubated at room temperature with 0.2 ng of a ^{32}P-labeled 148-bp DNA probe. Unlabeled supercoiled pRSVneo DNA (40 ng for hamster extracts and 2000 ng for human extracts) was added to each binding reaction mixture to titrate away the effects of nonspecific DNA-binding proteins.

Electrophoretic Mobility-Supershift Assay. To test the recognition of DEB factor by Ku antisera, binding reactions were done with the addition of human antisera HT and OM, which were diluted by factors of 10^3, 10^4, and 10^5 in 1% bovine serum albumin.

Fractionation of Cell Extracts. Nuclear extract from CHO AA8 cells was bound to heparin agarose, which was then poured into a column, washed with buffer [12 mM Hepes, pH 7.9/0.1 M NaCl/5 mM $MgCl_2$/0.6 mM dithiothreitol/0.6 mM EDTA/0.1 mM phenylmethanesulfonyl fluoride/12% (vol/vol) glycerol], and then eluted with the same buffer containing higher salt concentrations in steps of 0.1 M up to 1 M NaCl.

Western Blots. Nuclear extract proteins were resolved by SDS/PAGE, transferred to GSWP membrane (Millipore), and probed with OM and HT antisera followed by horseradish peroxidase-conjugated goat anti-human IgG (TAGO). Antibody binding was detected by enhanced chemiluminescence (Amersham).

RESULTS

X-ray sensitivity and DEB activity were strictly correlated in the three mutant group 5 cell lines xrs5, XR-V15B, and XR-V9B. All three lines are abnormally sensitive to x-rays and deficient in expressing DEB activity (19). When xrs5 cells were treated with the demethylating agent 5-azacytidine and selected with x-rays, pooled revertants demonstrated

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Abbreviations: DEB, DNA end-binding; DNA-PK, DNA-dependent protein kinase.

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wild-type x-ray survival (Fig. 1A). In parallel with x-ray resistance, DEB activity was absent from xrs5 cells and restored to wild-type levels in the xrs5 revertants (Fig. 1B). Similarly, when XR-V15B cells were treated with azacytidine and selected with x-rays, surviving colonies expressed wild-type levels of DEB activity.

In control experiments, bulk cultures of xrs5 and XR-V15B cells were treated with azacytidine but not selected with x-rays, and the treated cells contained no detectable DEB activity. Finally, to rule out an effect specific for azacytidine, cultures of xrs5 and XR-V15B cells were treated with x-ray selection alone. A small number of x-ray-resistant revertants were recovered (2–3 clones per 500,000 cells), and the revertants also expressed DEB activity. In this experiment, both the restoration of x-ray resistance and the restoration of

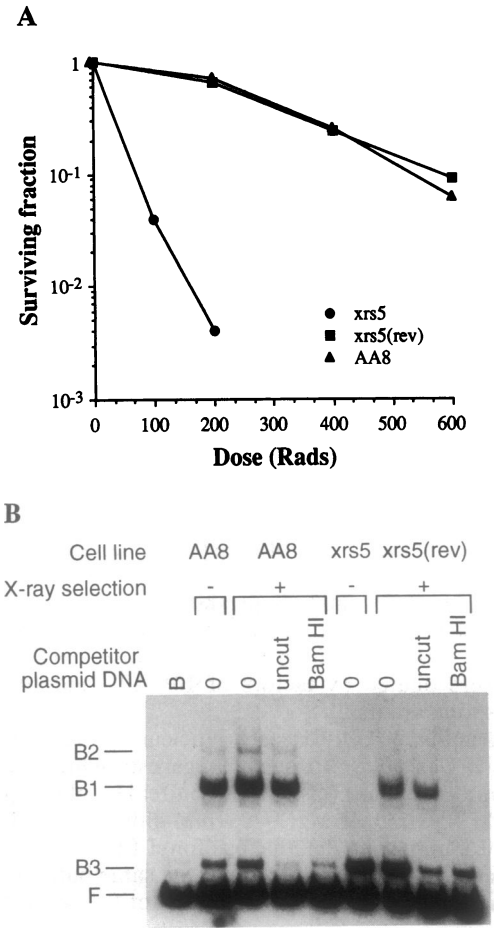

FIG. 1. (A) X-ray survival was restored in xrs5 revertant cells. Wild-type AA8 (▲), mutant xrs5 (●), and pooled revertant xrs5(rev) (■) cells were plated in triplicate in 10-fold dilutions and treated with x-ray doses up to 600 rads (1 rad = 0.01 Gy). Surviving cells were grown up as colonies, which were stained with 10% Giemsa reagent and counted. (B) DEB activity was restored in xrs5 revertant cells. Extracts from AA8, xrs5, and xrs5(rev) cells were assayed for DEB activity. In lanes marked uncut and *Bam*HI, 200 ng of competitor pRSVneo DNA was added as supercoiled plasmid (uncut) or as plasmid DNA cut once by *Bam*HI restriction endonuclease. In lanes marked 0, no additional competitor was added. The lane marked B was loaded with a binding reaction mixture from which extract was omitted. The pooled revertant xrs5(rev) cells were derived by treatment with 5-azacytidine (3 μg/ml) followed by x-ray selection with doses of 160 rads per day for four consecutive days (22). Therefore as a control, the AA8 cells were exposed to the same x-ray selection and found to contain the same amount of DEB activity. F indicates the position of free f148 DNA probe. B1 and B2 indicate binding activity specific for DNA ends, since cut plasmid DNA competed strongly for binding activity while uncut plasmid failed to compete. B3 indicates nonspecific binding activity.

DEB activity were infrequent events, making their simultaneous occurrence in two independently derived cell lines highly significant.

DEB factor had a number of striking similarities to Ku protein (Table 1). Both Ku and DEB factor bind to double-stranded DNA ends with 5′ or 3′ overhangs or blunt ends but not to the single-stranded ends from the homopolymers poly(dA) and poly(dT) (19, 23). Interestingly, both DEB and Ku binding activities (24) were Mg^{2+} independent (data not shown) and could be blocked by competition with single-stranded circular M13 virion DNA (19). More than two molecules of Ku can bind and load onto one DNA fragment, producing a ladder of mobility shifts (25). Similarly, DEB activity may be seen in bands B1 and B2 and at least one additional band above B2 (19). Both DEB factor and Ku were localized to the nucleus and moderately abundant. HeLa cells contain at least 200,000 molecules of DEB factor (19), if bands B1 and B2 represent binding by 1 and 2 molecules to the DNA probe. The actual abundance must be somewhat higher, since the binding assay was done with a 10,000-fold excess of competitor DNA, which titrates away some DEB activity. By comparison, HeLa cells contain about 400,000 molecules of Ku (6).

To further investigate the possible identity of DEB and Ku, two different human Ku antisera, OM and HT, were added to the binding reaction mixtures containing cell extract and DNA probe. OM is known to recognize both the 86- and 70-kDa subunits of Ku, whereas HT recognizes only the 70-kDa subunit (26). Both antisera produced a supershifted mobility, accompanied by a decrease in DEB activity, when added to binding reaction mixtures with either hamster or human extracts (Fig. 2A). This effect was consistent with binding of Ku antibodies to the complex of DEB factor and the DNA probe. To demonstrate the dependence of the supershift on DEB activity, *Bam*HI-cleaved plasmid DNA was added to the binding reaction mixtures, causing both the DEB activity and the supershift to disappear. Furthermore, no supershift was observed in the XR-V15B extract, which lacks DEB activity. In controls for specificity of the supershift, no effect was seen when normal human serum was tested on extracts from the three cell lines (data not shown) or when the Ku antisera were tested against an unrelated DNA-binding protein, xeroderma pigmentosum group E binding factor (XPE-BF), which binds to DNA damaged by ultraviolet radiation (27–29) (Fig. 2B). Thus, DEB factor and Ku are antigenically related, if not identical.

To detect the expression of Ku-related polypeptides, Western blots were probed with OM and HT antisera. Both antisera identified a 70-kDa polypeptide in hamster nuclear extract (Fig. 3). The OM antiserum, which recognizes human Ku p86 as well as p70, failed to recognize a hamster protein at 86 kDa. In a control Western blot, normal human serum failed to react with the 70-kDa polypeptide (data not shown). Thus, the 70-kDa hamster polypeptide was of the same molecular mass and antigenically similar if not identical to the Ku p70 subunit.

Most significantly, the hamster 70-kDa polypeptide detected by OM antiserum was severely deficient in extracts of

Table 1. Comparison of Ku and DEB factor

	Ku	DEB factor
Cellular localization	Nuclear	Nuclear
Abundance, molecules per HeLa cell	400,000	>200,000
Mg^{2+}-independent DNA binding	Yes	Yes
DNA substrates		
Double-stranded DNA ends (5′, 3′, and blunt)	Yes	Yes
Homopolymers [poly(dA) or poly(dT)]	No	No
M13 circular virion DNA	Yes	Yes

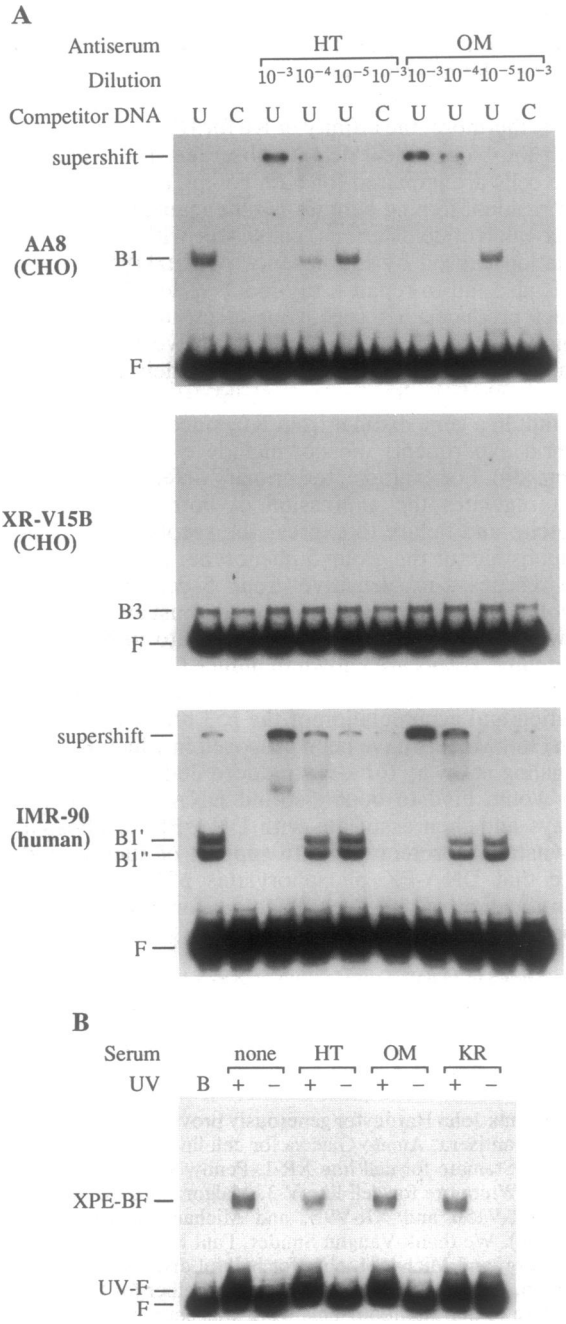

FIG. 2. (*A*) Ku antisera supershifted DEB activity. Ku antisera HT and OM were added to binding reaction mixtures for wild-type hamster AA8, mutant XR-V15B, and wild-type human IMR-90 cells in dilutions of 10^{-3}–10^{-5}. The binding reaction mixtures contained competitor plasmid DNA either uncut (U) or cut (C) with *Bam*HI. The DEB activities in AA8 and IMR-90 extracts were supershifted to the loading well of the gel by the antisera. F indicates the position of free f148 DNA probe. Note that the pattern of DEB activity was different for the human IMR-90 cells (B1′ and B1″). However, in other human cell lines, including HeLa and Jurkat, the pattern of DEB activity was the same as that found in the hamster AA8 cells (unpublished data). (*B*) Ku antisera did not supershift the unrelated DNA-binding activity xeroderma pigmentosum group E binding factor (XPE-BF). The Ku antisera (HT and OM) and normal serum (KR) were added to binding reaction mixtures designed to reveal XPE-BF (27), a binding activity specific for the same f148 DNA probe damaged by ultraviolet radiation (UV-F). B denotes a binding reaction omitting extract.

xrs5 and XR-V15B, the same cell lines which lacked DEB activity, and was partially deficient in XR-V9B extract, in which DEB activity was partially deficient (Fig. 3). The

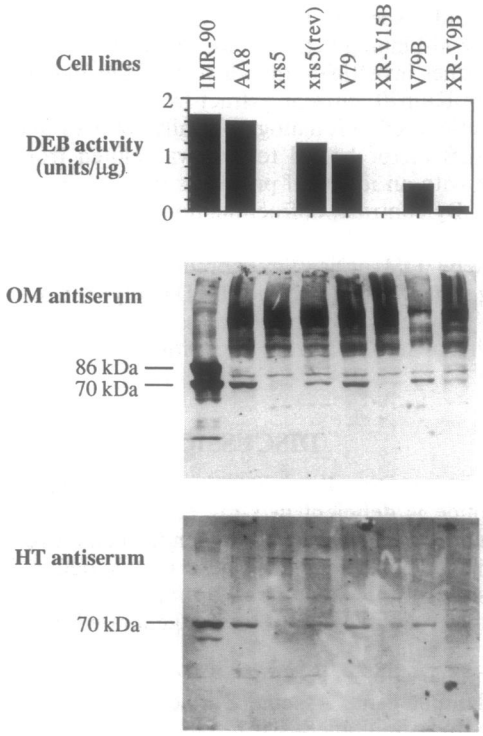

FIG. 3. A 70-kDa polypeptide antigenically related to Ku p70 was deficient in x-ray-sensitive CHO mutants deficient in DEB activity. (*Top*) DEB activity in wild-type human cells (IMR-90), CHO mutant cells (xrs5, XR-V15B, and XR-V9B), the corresponding wild-type cell lines (AA8, V79, and V79B, respectively), and the pooled revertants [xrs5(rev)] as determined by scanning densitometry (19). One unit of binding activity is defined as a shift of 30% of the labeled probe. (*Middle* and *Bottom*) Western blots of nuclear extracts (30 μg protein per lane) probed with Ku antisera OM and HT.

70-kDa polypeptide was restored to normal levels in the pooled xrs5 revertants (Fig. 3), as well as XR-V15B and XR-V9B revertants (data not shown).

The results with OM antiserum were confirmed with HT. The hamster 70-kDa polypeptide was clearly deficient in xrs5 and XR-V15B, partially deficient in XR-V9B, and restored in xrs5 revertants (Fig. 3). HT did detect a very weak band at 70 kDa in xrs5 and XR-V15B, and it is possible that this band represents residual expression of the 70-kDa polypeptide also recognized by OM. However, it may also be due to a second protein of the same molecular mass that happens to cross-react with HT, although OM detects the 70-kDa polypeptide more strongly than HT but detects less background activity in xrs5 and XR-V15B.

The XR-V9B cells appeared to have an intermediate phenotype. They are less sensitive to x-rays than xrs5 or XR-V15B cells (14) and express residual DEB activity and residual levels of the 70-kDa polypeptide (Fig. 3). To further explore the genetic basis of this phenotype, we isolated clonal populations of XR-V9B. Thirteen of 15 clones contained no detectable DEB activity, while the remaining 2 clones expressed wild-type levels of DEB activity and were fully x-ray resistant. Thus, XR-V9B is a heterogeneous population, probably due to the emergence of spontaneous revertants during passage.

The 70-kDa polypeptide was expressed at normal levels in other cell lines sensitive to x-rays and deficient in V(D)J recombination: hamster XR-1, hamster V3, and mouse scid/St. It was also normal in the x-ray-sensitive cell lines hamster EM9 and human AT-5BI (ataxia–telangiectasia group D). All of these cell lines contained normal levels of DEB activity (19). Thus, the 70-kDa polypeptide is antigenically similar to

Ku p70 and is specifically deficient in the same cell lines that lack an end-binding activity biochemically similar to Ku.

To further investigate the identity of the 70-kDa polypeptide with Ku p70, nuclear extract from wild-type CHO cells was fractionated chromatographically. In a 10-fold purification, DEB factor bound to and was eluted from heparin-agarose with an identical profile as the 70-kDa polypeptide (Fig. 4). By contrast, cofractionation did not occur between DEB activity and the higher molecular weight proteins recognized by OM antiserum, nor did it occur between the binding activity in B3 and the 70-kDa polypeptide. Thus, the 70-kDa polypeptide cofractionates with an end-binding activity similar to Ku, further suggesting that it is identical to Ku p70.

DISCUSSION

In conclusion, the Ku p70 subunit or a closely related polypeptide is deficient in x-ray complementation group 5 cells and is invariably restored when the cells revert to x-ray resistance. These results suggest that a defect in Ku is responsible for the phenotype of group 5, leading to impaired resolution of double-strand breaks generated by x-rays and V(D)J recombination.

The primary defect in group 5 cells may be in either the Ku p70 or Ku p86 gene. Assembly of active Ku protein *in vitro* requires the simultaneous translation of both properly encoded subunits (30). Mixing experiments in which each protein was translated individually failed to reconstitute DNA-binding activity. Thus, a mutation in either gene could lead to denaturation and subsequent *in vivo* degradation of the protein subunit for the other gene.

In fact, data suggest that mutation in the Ku p86 gene may be the primary defect in group 5 cells. The human Ku p70 gene maps to chromosome 22q13, whereas the Ku p86 gene maps to chromosome 2q33–35 (31). X-ray resistance in group 5 cells was partially restored in hamster–human hybrids by human chromosome fragments mapping to 2q35 (32, 33), in striking concordance with the location of the Ku p86 gene. By contrast, human chromosomes 5, 6, 9, 12q, 13, 15, 17, and 21 did not rescue group 5 cells (34).

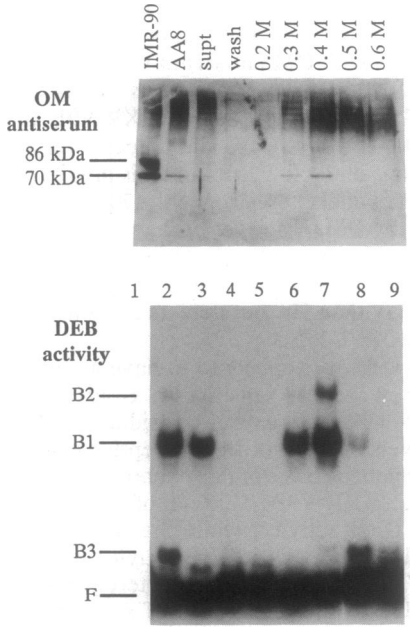

FIG. 4. The 70-kDa polypeptide cofractionated with DEB activity. Nuclear extract from CHO AA8 cells was fractionated on a heparin-agarose column. The supernatant from binding to heparin-agarose (supt), the wash, and the elution fractions were assayed for Ku-related proteins by Western blot with OM antiserum and for DEB activity by the electrophoretic mobility-shift assay. The Western blot also includes nuclear extract from human IMR-90 cells to show the position of the 70- and 86-kDa subunits of Ku.

Furthermore, the affinity of Ku for DNA ends is consistent with the biochemical defects observed in the mutant cells. The cells are impaired for both coding and signal joins, and the residual joining activity is characterized by abnormally large nucleotide deletions, consistent with exonuclease degradation caused by the absence of Ku/DEB activity. The cells also fail to repair x-ray-induced double-strand breaks, which produce a heterogeneous spectrum of DNA ends (35). This DNA repair pathway might involve Ku, which indeed binds to DNA ends independently of their structure.

Nevertheless, it remains possible that group 5 cells are mutant in a gene distinct from Ku, since the hamster–human hybrid experiments did not include every human chromosome (34). For example, the primary defect could be in a gene that regulates the expression of both Ku and a second protein, and failure to express the second protein might be responsible for the group 5 phenotype. Definitive proof that Ku rescues x-ray-sensitive group 5 cells and is therefore involved in recombination and repair must await experiments in which Ku cDNAs are transfected into the mutant cells.

Finally, x-rays are known to induce G_1 cell cycle arrest by a pathway that is mediated by the posttranscriptional stabilization and accumulation of the p53 tumor-suppressor protein (36). Models have been proposed in which Ku initiates a signaling pathway for x-ray-induced cell cycle arrest. Thus, Ku would bind to double-strand DNA breaks induced by x-rays and then associate with DNA-PK to phosphorylate downstream proteins (37). In support of such a model, we note that DNA-PK phosphorylates p53 *in vitro* (9). The identification of cell lines defective for Ku will allow such models to be tested *in vivo*.

Note Added in Proof. Transfection of XR-V15B cells with an expression vector for Ku p86 cDNA conferred DEB activity, x-ray resistance, and V(D)J recombination activity, while transfection with Ku p70 cDNA had little or no effect (V. Smider, W.K.R., M. Lieber, and G.C., unpublished work).

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