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Ku86 Defines the Genetic Defect and Restores X-Ray Resistance and V(D)J Recombination to Complementation Group 5 Hamster Cell Mutants

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X-ray-sensitive hamster cells in complementation groups 4, 5, 6, and 7 are impaired for both double-strand break repair and V(D)J recombination. Here we show that in two mutant cell lines (XR-V15B and XR-V9B) from group 5, the genetic defects are in the gene encoding the 86-kDa subunit of the Ku autoantigen, a nuclear protein that binds to double-stranded DNA ends. These mutants express Ku86 mRNA containing deletions of 138 and 252 bp, respectively, and the encoded proteins contain internal, in-frame deletions of 46 and 84 amino acids. Two X-ray-resistant revertants of XR-V15B expressed two Ku86 transcripts, one with and one without the deletion, suggesting that reversion occurred by activation of a silent wild-type allele. Transfection of full-length cDNA encoding hamster Ku86 into XR-V15B cells resulted in a complete rescue of DNA-end-binding (DEB) activity and Ku70 levels, suggesting that Ku86 stabilizes the Ku70 polypeptide. In addition, cells expressing wild-type levels of DEB activity were fully rescued for X-ray resistance and V(D)J recombination, whereas cells expressing lower levels of DEB activity were only partially rescued. Thus, Ku is an essential component of the pathway(s) utilized for the resolution of DNA double-strand breaks induced by either X rays or V(D)J recombination, and mutations in the *Ku86* gene are responsible for the phenotype of group 5 cells.

All cells possess a mechanism for repairing DNA double-strand breaks (DSBs) produced by ionizing radiation. Cells of the immune system must also resolve DNA DSBs produced by V(D)J recombination of the immunoglobulin and T-cell receptor genes during development of B and T cells (reviewed in reference 28). In fact, the biochemical pathways for DSB repair and V(D)J recombination have a number of common factors. Evidence of this was first described for the severe combined immune deficient (*scid*) mouse, which is hypersensitive to ionizing radiation because of defective DSB repair (1, 12, 18) and immune deficient because of defective V(D)J recombination (29). Subsequently, other X-ray-sensitive rodent cell lines defective in DSB repair were also found to have impaired V(D)J recombination (17, 27, 34, 45, 47). These cell lines fall into four complementation groups, 4, 5, 6, and 7, with group 7 corresponding to the *scid* defect (43, 52). The human genes capable of rescuing these mutants are designated *XRCC4*, *XRCC5*, *XRCC6*, and *XRCC7*, respectively (*XRCC* denotes X ray cross-complementing) (for a review, see reference 51).

Recently, it has been shown that the Ku protein is involved in both DSB repair and V(D)J recombination. Ku is an abundant nuclear protein identified originally as an autoantigen recognized by sera from patients with autoimmune diseases, including scleroderma-polymyositis overlap syndrome and systemic lupus erythematosus (31). Ku is a heterodimer of two tightly associated polypeptides of 70 and 86 kDa (Ku70 and Ku86, respectively) encoded by genes which have been mapped in humans to chromosomes 22q13 and 2q33-35, respectively (4, 32, 38). Biochemically, it has been shown that Ku binds to double-stranded DNA ends, nicks, gaps, and DNA hairpins (30). It also forms a complex with a catalytic subunit of 450 kDa to form the DNA-dependent protein kinase. In vitro, the DNA-dependent protein kinase phosphorylates several proteins, such as RNA polymerase II, Sp1, p53, c-Fos, c-Jun, c-Myc, and Ku itself (20). The gene encoding the catalytic subunit (14) maps to human chromosome 8q11 (19, 24–26) and has recently been shown to rescue the *scid* defect (2, 23, 35).

There is indirect evidence that mutation of the *Ku86* gene might be the primary defect in complementation group 5 mutants. The *XRCC5* gene has been mapped to 2q35, the same region as that of the *Ku86* gene (6, 16, 21). In addition, group 5 cells lack a DNA-end-binding (DEB) activity with an abundance, nuclear localization, DNA substrate specificities, and antigenic determinants similar to those of Ku (13, 36, 37). Transfection of the human Ku86 cDNA into group 5 cells partially restores DEB activity, X-ray resistance, and V(D)J recombination activity in different mutants belonging to this group (3, 42, 44).

Nevertheless, direct evidence defining the primary defect in mutants of group 5 was still lacking. First, human Ku86 cDNA did not completely rescue group 5 mutants. Second, chromosome mapping experiments sampled only a fraction of the human genome (6, 21) and therefore failed to rule out the possibility that complementing activity was present on another chromosome in addition to chromosome 2. Therefore, we in-

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troduced a wild-type hamster cDNA into group 5 mutant cells and demonstrated that this could completely restore DEB activity, Ku70 protein levels, X-ray resistance, and V(D)J recombination. Furthermore, we isolated cDNAs encoding Ku86 from two independently derived group 5 mutants and demonstrated that these cDNAs are mutated. These results unequivocally demonstrate that mutation of Ku86 is directly responsible for the defects exhibited by XRCC5 cells and confirm the idea that Ku86 is a critical component of the mammalian DNA DSB repair pathway.

MATERIALS AND METHODS

Cell culture. XR-V15B and XR-V9B mutant cell lines derived from Chinese hamster V79 and V79B cells, respectively, have been described previously (53, 54). All cell lines were cultured in Ham's F-10 medium (without hypoxanthine and thymidine) supplemented with 15% newborn calf serum or in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% L-glutamine. Culture media also included penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Cells were maintained at 37°C in a 5% CO_2 atmosphere, humidified to 95 to 100%.

To isolate X-ray-resistant revertants, XR-V15B and a thioguanine- and ouabain-resistant derivative (XR-V15BTOR) cell line were seeded (at 10^6 cells per 10-cm-diameter plate) and exposed to an X-ray dose of 2 Gy for three consecutive days. Clones of cells surviving this selection were isolated and examined for X-ray survival. Two revertants (XR-V15B-Rev1 and XR-V15B-Rev2) from XR-V15B and one revertant (XR-V15B-Rev3) from XR-V15BTOR showed wild-type X-ray survival.

DNA transfection. Syrian hamster Ku86 cDNA inserted into the pSP65-SRα vector under the control of the SRα promoter (46) or the same vector lacking this insert (10 μg) was cotransfected with pRSVneo (2 μg) and carrier DNA consisting of pUC19 (8 μg) by coprecipitation with calcium phosphate in 0.8 ml onto a 10-cm-diameter plate containing 10^6 cells (15). After 48 h, cells were passaged and then selected for 14 days in medium containing G418 (Gibco, Gaithersburg, Md.) at 400 μg/ml. Colonies stably resistant to G418 were isolated and characterized as described below.

Electrophoretic mobility shift assay. Nuclear extracts were prepared by the Nonidet P-40 lysis method previously described (36). Extract (0.6 μg) was incubated with radiolabeled f148 probe in the presence of closed circular plasmid DNA to mask the effect of nonspecific DNA-binding proteins. Protein-DNA complexes were resolved on a nondenaturing 5% polyacrylamide gel.

Immunoblotting. Nuclear extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane (GSWP; Millipore, Bedford, Mass.), and probed with human OM antiserum (a kind gift from John Hardin, 1:2,000 dilution) followed by horseradish peroxidase-conjugated goat antibody to human immunoglobulin G (TAGO, Burlingame, Calif.). Antibody binding was detected by enhanced chemiluminescence (Amersham, Arlington Heights, Ill.).

X-ray survival. G418-resistant clones were plated in triplicate and X-irradiated at doses ranging from 0 to 6 Gy. After 14 days, surviving colonies were stained with 10% Giemsa stain and counted. Each survival curve represents the average of two independent experiments.

V(D)J recombination. G418-resistant clones were removed from G418 on the day prior to beginning the assay. Expression plasmids for RAG-1 and RAG-2 (8 μg each) were transiently transfected with either pJH290 (4 μg) to test for coding joint formation or pJH200 (4 μg) to test for signal joint formation. DNA was transfected by coprecipitation with calcium phosphate. After 48 h, DNA was harvested, digested with *Dpn*I to eliminate unreplicated plasmids, and electroporated into *Escherichia coli*. Unrearranged plasmids conferred resistance to ampicillin (Amp^r), whereas rearranged plasmids conferred resistance to both ampicillin and chloramphenicol (Amp^r Cam^r). Recombination frequency is expressed as the ratio of doubly drug-resistant colonies to the number of ampicillin-resistant colonies. Valid V(D)J recombination events were confirmed by digesting plasmids recovered from *E. coli* with *Apa*LI (which cleaves at the signal joint) or *Pvu*II (which releases a fragment spanning a correctly formed coding joint) (34).

RT-PCR analysis of hamster Ku86 mRNAs from wild-type and mutant cells. Total RNA was isolated by the guanidium isothiocyanate method previously described (7). First-strand Ku86 cDNA synthesis was performed as follows: total RNA (1 μg) was added to a reverse transcription (RT) solution consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM (each) deoxynucleoside triphosphates, 2.5 μM oligo$(dT)_{15}$ primer, 20 U of RNasin, and 50 U of murine leukemia virus reverse transcriptase (Geneamp RNA PCR kit). The reaction mixture (final volume, 20 μl) was incubated for 30 min at 42°C, heated to 95°C for 5 min, and then chilled on ice. The newly generated RNA-cDNA hybrids were amplified by PCR with Ku86-specific primers Ku-5 and Ku-3b, resulting in a 2.3-kb product. PCR was performed with cycles consisting of denaturation for 30 s at 94°C, primer annealing at 55°C for 30 s, and extension at 72°C for 2 min (DNA thermal cycler; Perkin-Elmer Cetus, Norwalk, Conn.).

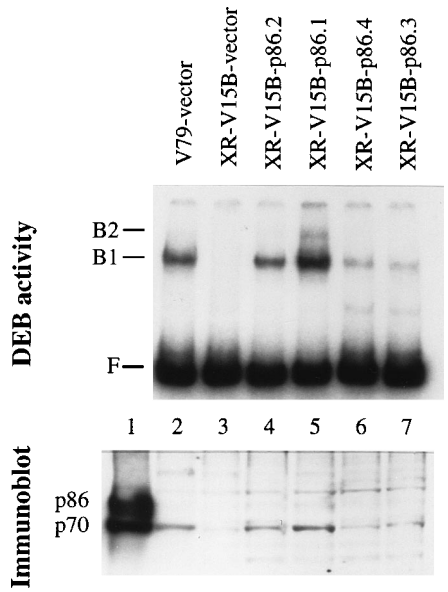

FIG. 1. Expression of Ku in wild-type V79 and mutant XR-V15B hampster cells transfected with Ku86. (Top) DEB activity. The levels of DEB activity in one clone isolated from the wild-type hamster cell line V79 transformed with vector alone (V79-vector; lane 2), one clone isolated from the transfection of XR-V15B with vector alone (XR-V15B-vector; lane 3), and four clones of mutant XR-V15B cells transfected with hamster Ku86 (XR-V15B-p86.1, XR-V15B-p86.2, XR-V15B-p86.3, and XR-V15B-p86.4; lanes 4 to 7) are shown. Clones are numbered in order of decreasing DEB activity. F, positions of free DNA probe; B1 and B2, positions of protein-DNA complexes consisting of DEB activity and the DNA probe. (Bottom) Immunoblot for expression of Ku70. The immunoblot was probed with OM antiserum, which reacts strongly with both Ku86 and Ku70 in human cells and less strongly with Ku70 in hamster cells. The expression of Ku70 in the hamster cell lines used for the upper panel is shown. Lane 1 is a control with HeLa extract to show the mobilities of human Ku70 and Ku86 (p70 and p86, respectively).

DNA sequence analysis. Primer Ku-3b was biotinylated, and after PCR, the amplified 2.3-kb cDNA was gel purified (Qiaex; Qiagen, Chatsworth, Calif.). Magnetic M-280 Dynabeads (Dynal AS, Oslo, Norway) were used to prepare single-stranded, immobilized templates. The DNA sequence was obtained by using a T7 sequence kit (Pharmacia Biotech, Uppsala, Sweden) with [α-^{32}P] dATP. Samples were resolved on a 6% polyacrylamide gel at 40 W. Gels were exposed to X-ray film. Sequence analysis was also performed after cloning the PCR products in a TA cloning vector (Promega, Madison, Wis.). The primer sequences were as follows: Ku-2, 5′-GGAGGAGGCCATCCAGTT-3′; Ku-3b, 5′-ATGGCTTCCAGGATGCTCTTTC-3′; Ku-4, 5′-TGATGCTACCAGATTTTG-3′; Ku-5, 5′-AAGTAACCAAACCGCCCGTG-3′; Ku-6, 5′-ATTGTAGCCTATAAATCG-3′; Ku-7, 5′-CCGTAGCGGAACCCTTGAAT-3′; Ku-8, 5′-AGAGCTAATCCTCAAGTTGG-3′; Ku-8b, 5′-GGTTGAACTCCTAATCGAGA-3′; Ku-9, 5′-ACTCCATCCTGAACAACAAT-3′; Ku-10, 5′-ATTCAGCAGCACATTTTG-3′; Ku-12, 5′-CAAAATGTGCTGCTGAAT-3′; and Ku-13, 5′-CTTTGCAGCAAAAGATGAT-3′.

Nucleotide sequence accession numbers. The Chinese hamster Ku86 sequence has been deposited in the GenBank database under accession number L48606. The GenBank and EMBL accession numbers for the Syrian hamster, mouse, and human Ku86 nucleotide sequences are U40570, X66323, and M30938, respectively.

RESULTS

Restoration of DEB activity. A hamster cDNA encoding Ku86 had been cloned previously from a Syrian hamster liver library (17). Therefore, XR-V15B mutant cells were transfected either with expression vectors containing the Syrian hamster Ku86 or with a vector lacking this insert. As a control, the parental, wild-type V79 cell line was also transfected with a vector lacking this insert. Stable G418-resistant clones were isolated and analyzed for DEB activity. Of 12 clones selected in G418 from XR-V15B cells transfected with Ku86, 5 expressed

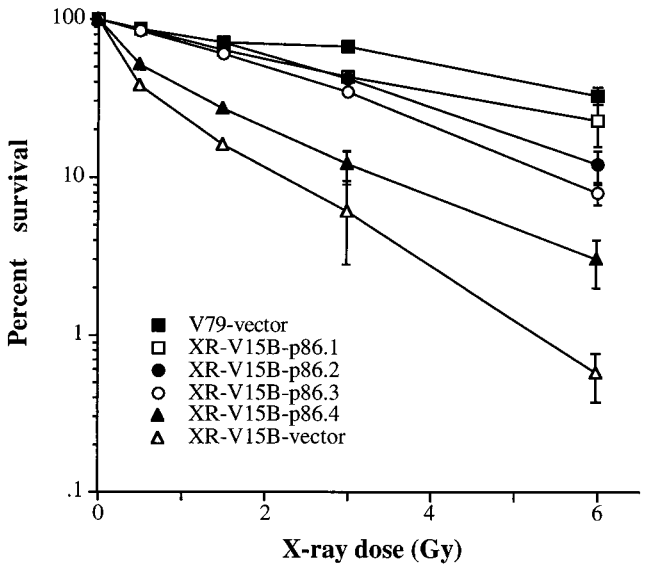

FIG. 2. X-ray survival of XR-V15B cells transfected with hamster Ku86 cDNA. Clones are labeled as in Fig. 1. Single cells were plated at low density and X-irradiated at various doses, and then the percentages of cells surviving to form colonies at 14 days postirradiation were determined. Data are the averages of two experiments, each performed in triplicate. Vertical bars show standard deviations.

detectable DEB activities. Four of these clones were chosen for further analysis because they showed a range of DEB activities, from below to above wild-type levels (Fig. 1). The clones were designated according to their levels of DEB activity from highest to lowest, XR-V15B-p86.1, XR-V15B-p86.2, XR-V15B-p86.3, and XR-V15B-p86.4. Of the nine clones isolated from XR-V15B cells transfected with vector alone, none showed DEB activity. Thus, the probability of finding spontaneous revertants among transfected XR-V15B cells was too low to account for the five clones with detectable DEB activities.

Restoration of Ku70 protein level. It was previously shown that group 5 cells lack Ku70 protein, even though the group 5 defect did not map to the same chromosome as that of the *Ku70* gene (37, 42). Therefore, we investigated the status of Ku70 expression in stably transfected clones. Complete restoration of Ku70 protein to wild-type levels was observed by immunoblot with Ku antiserum in the clone expressing the highest level of DEB activity (XR-V15B-p86.1) (Fig. 1). Smaller increases in Ku70 protein levels were seen for clones which expressed lower levels of DEB activity (XR-V15B-p86.2, XR-V15B-p86.3, and XR-V15B-p86.4). Very little, if any, detectable Ku70 protein was detected in a cloned line transfected with vector alone (XR-V15B-vector). Thus, the introduction of Ku86 cDNA to XR-V15B cells restored Ku70 accumulation.

Complementation of X-ray sensitivity. Previous studies demonstrated that the introduction of human Ku86 cDNA into the hamster XR-V15B cell line partially complemented its X-ray sensitivity (42). To test if complete complementation could be obtained with hamster cDNA, we tested our transfected clones for X-ray resistance. Complete restoration of X-ray resistance was attained in clone XR-V15B-p86.1, which expressed the highest level of DEB activity (Fig. 2). Only partial, albeit significant, restoration of X-ray resistance was seen in clones expressing lower levels of DEB activity (Fig. 2). Thus, hamster Ku86 cDNA can fully complement the X-ray sensitivity of group 5 cells; this is dependent on the level of DEB activity.

Complementation of V(D)J recombination. Group 5 cells are defective in forming coding and signal joints during V(D)J recombination (34, 45). Therefore, we tested our transfected clones for proficiency in V(D)J recombination by measuring both signal and coding joint formation in an extrachromosomal assay (42). V(D)J recombination was measured for both signal and coding joint formation, and valid recombination events were confirmed by restriction analysis. Clones expressing the highest levels of DEB activity also had the highest levels of V(D)J recombination activity (Fig. 3). Within the expected uncertainty of this assay, clone XR-V15B-p86.1 showed wild-type or nearly wild-type levels of V(D)J recombination. Interestingly, in each of the partially rescued clones, the relative restoration of coding joint formation was less efficient than that of signal joint formation. This suggests that full Ku expression is more important for coding joint formation. In summary, the introduction of hamster Ku86 cDNA into XR-V15B cells restored DEB activity, Ku70 expression, X-ray sensitivity, and V(D)J recombination. The level of restoration in each case

Cell Line	Amp^R	Amp^RCam^R	RF (10^{-3})
	Signal joints		
V79-vector	31800	32	1.01
V15B-p86.1	55000	44	.80
V15B-p86.2	79900	42	.53
V15B-p86.3	77500	34	.44
V15B-p86.4	80400	34	.43
V15B-vector	86600	0	<.01
	Coding joints		
V79-vector	9335	60	6.43
V15B-p86.1	13480	60	4.45
V15B-p86.2	36800	48	1.30
V15B-p86.3	14000	24	1.71
V15B-p86.4	18500	8	.43
V15B-vector	46600	0	<.02

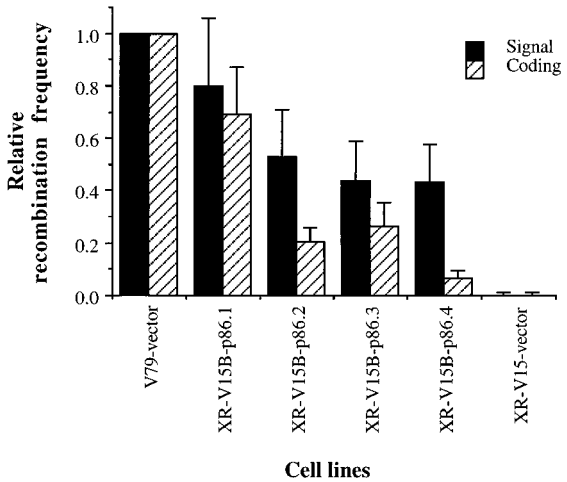

FIG. 3. V(D)J recombination activity of XR-V15B cells transfected with hamster Ku86 cDNA. Clones are labeled as in Fig. 1. Cell lines were transfected with the *RAG-1* and *RAG-2* genes along with a substrate plasmid (see Materials and Methods). The V(D)J recombination frequency (RF) was measured for both signal and coding joints as the number of doubly resistant (Amp^r Cam^r) colonies divided by the number of Amp^r colonies. Vertical bars show standard deviations.

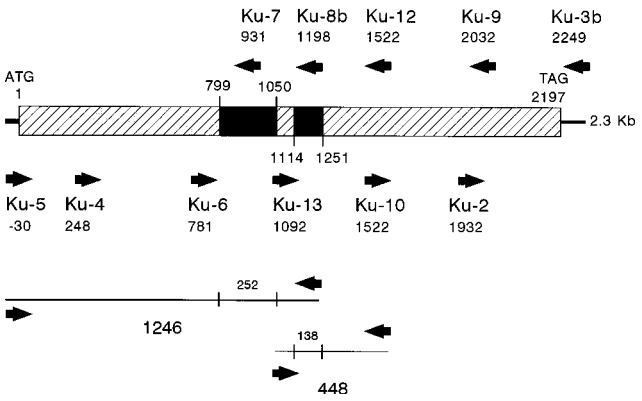

FIG. 4. Hamster Ku86 cDNA and PCR primers. The positions of the primers used for sequencing and RT-PCR are indicated by arrows. Black boxes correspond to regions deleted in the Ku86 cDNAs of mutants XR-V9B and XR-V15B.

correlated with the level of DEB activity observed, suggesting that Ku was limiting in abundance for all these processes.

Nucleotide sequence of the Chinese hamster Ku86 cDNA. To study the molecular nature of the defects in XR-V15B and XR-V9B, another X-ray-sensitive Chinese hamster mutant belonging to complementation group 5 (53, 54), the cDNA of Ku86 from parental cell line V79B was sequenced. Total V79B RNA was used to synthesize oligo(dT)-primed cDNA by murine leukemia virus reverse transcriptase. Subsequently, this cDNA was amplified by PCR with Ku86-specific primers Ku-5 and Ku-3b, which were based on the sequence of Syrian hamster Ku86 cDNA (Fig. 4). By using a series of primer walking steps, the resulting 2.3-kb PCR fragment containing the entire open reading frame of Ku86 was sequenced.

The nucleotide and predicted amino acid sequences of the Chinese hamster cDNA of Ku86 are presented in Fig. 5. The Chinese hamster Ku86 cDNA sequence is 95.5, 87.4, and 80.3% identical to the Syrian hamster (17), mouse (10), and human (32, 50) homologs, respectively (Fig. 6).

Sequence analysis of Ku86 cDNAs from XR-V15B and XR-V9B mutants. To determine whether the Ku86 cDNAs in the X-ray-sensitive mutants XR-V15B and XR-V9B were abnormal, PCR-amplified Ku86 cDNA was sequenced as described in Materials and Methods. Comparison of these sequences with the wild-type Ku86 sequence revealed a deletion of 138 bp from nucleotides +1114 through +1251 in the XR-V15B cDNA and a deletion of 252 bp from +799 through +1050 in

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-30  aag taa cca aac cgc ccg tgg acc agc aac ATG GCG TGG TCC GCT AAT AAG GCA GCT GTT GTG CTG
  1                                         M   A   W   S   A   N   K   A   A   V   V   L
 37  TGT ATG GAT GTG GGC GTT GCT ATG GGT AAC TCT TTT CCT GGC GAA GAA TCC TCG TTT GAA CAA GCA
 13   C   M   D   V   G   V   A   M   G   N   S   F   P   G   E   E   S   S   F   E   Q   A
103  AAG AAA GTG ATG ACT ATG TTT GTC CAA CGA CAG GTG TTT TCT GAG AGC AAG GAT GAG ATT GCC TTG
 35   K   K   V   M   T   M   F   V   Q   R   Q   V   F   S   E   S   K   D   E   I   A   L
169  GTC CTC TTT GGA ACA GAC AAC ACT AAC AAC GCC CTT GCT AGT GAG GAC CAG TAT CAG AAC ATC ACA
 57   V   L   F   G   T   D   N   T   N   N   A   L   A   S   E   D   Q   Y   Q   N   I   T
235  GTG CAC AGA CAC CTG ATG CTA CCA GAT TTT GAT TTG CTG GAA GAC ATC GAA AGC AAA ATC CAA CTA
 79   V   H   R   H   L   M   L   P   D   F   D   L   L   E   D   I   E   S   K   I   Q   L
301  GGC TCT CGA CAA GCT GAC ATC TTG GAC GCC CTG ATT GTG TGC ATG GAT TTG ATT CAG CGT GAA ACC
101   G   S   R   Q   A   D   I   L   D   A   L   I   V   C   M   D   L   I   Q   R   E   T
367  ATA GGG AAG AAG TTT GAG AAG AAG CAT ATT GAA GTG TTC ACT GAC CTC AGC AGC CCA TTC AGC CAA
123   I   G   K   K   F   E   K   K   H   I   E   V   F   T   D   L   S   S   P   F   S   Q
433  GAT CAA CTG GAT GTT ATC ATT TGT AAC TTG AAG AAG TCT GGC ATC TCC CTG CAG TTC TTC CTG CCT
145   D   Q   L   D   V   I   I   C   N   L   K   K   S   G   I   S   L   Q   F   F   L   P
499  TTT CCA ATC AGC AAG AAC GAT GAG ACT GGG GAC AGA GGA GAT GGT GAC TTG GGC TTG GAC CAC TGT
167   F   P   I   S   K   N   D   E   T   G   D   R   G   D   G   D   L   G   L   D   H   C
565  GGA CCC TCC TTT CCT CAA AAA GGA ATT ACT GAG CAG CAA AAG GAA GGC ATC TGC ATG GTG GAG AGG
189   G   P   S   F   P   Q   K   G   I   T   E   Q   Q   K   E   G   I   C   M   V   E   R
631  GTG ATG GTG TCT TTA GAA GGT GAA GAT GGG CTG GAT GAG ATC TAC TCC TTC AGT GAG AGT CTA CGG
211   V   M   V   S   L   E   G   E   D   G   L   D   E   I   Y   S   F   S   E   S   L   R
697  CGG CTG TGT GTC TTT AAG AAG ATT GAG AGG CGC TCC ATG CCC TGG TCC TGC CAG CTG ACC ATC GGC
233   R   L   C   V   F   K   K   I   E   R   R   S   M   P   W   S   C   Q   L   T   I   G
763  CCT GAT TTG TCT ATA AAG ATT GTA GCC TAT AAA TCG ATC GTA CAG GAG AAA GTT AAA AAG AGC TGG
255   P   D   L   S   I   K   I   V   A   Y   K   S   I   V   Q   E   K   V   K   K   S   W
829  ATA GTT GTG GAT GCA AGA ACT CTA AAG AAG GAA GAT ATA AGA AAA GAA ACT GTC TAT TGC TTA AAC
277   I   V   V   D   A   R   T   L   K   K   E   D   I   R   K   E   T   V   Y   C   L   N
895  GAT GAT GAC GAA ACT GAA GTT TCC AAA GAG GAC ACT ATT CAA GGG TTC CGC TAC GGA AGT GAT ATA
299   D   D   D   E   T   E   V   S   K   E   D   T   I   Q   G   F   R   Y   G   S   D   I
961  ATT CCT TTT TCT AAA GTG GAT GAG GAA CAA ATG AAA TAT AAA TCA GAG GGG AAG TGC TTC TCT GTT
321   I   P   F   S   K   V   D   E   E   Q   M   K   Y   K   S   E   G   K   C   F   S   V
1027 TTG GGA TTC TGT AGA TCT TCT CAG GTC CAC AGG AGA TTC TTT ATG GGA TAT CAA GTT CTG AAG GTC
343   L   G   F   C   R   S   S   Q   V   H   R   R   F   F   M   G   Y   Q   V   L   K   V
1093 TTT GCA GCA AAA GAT GAT GAG GCA GCG GCT GTT GCT CTC TCT TCC CTT ATT CAT GCT TTG GAT GAA
365   F   A   A   K   D   D   E   A   A   A   V   A   L   S   S   L   I   H   A   L   D   E
1159 TTA AAC ATG GTC GCC ATC GTT CGA TAT GCT TAT GAC AAA AGA GCT AAT CCT CAA GTT GGT GTA GCC
387   L   N   M   V   A   I   V   R   Y   A   Y   D   K   R   A   N   P   Q   V   G   V   A
1225 TTT CCT TAT ATT AAG GAT TCC TAT GAG TGT TTA GTT TAT GTG CAG CTG CCT TTC ATG GAA GAC TTG
409   F   P   Y   I   K   D   S   Y   E   C   L   V   Y   V   Q   L   P   F   M   E   D   L
1291 CGG CAA TAC ATG TTT TCA TCA CTG AAA AAC AAT AAG AAA TGC ACT CCC ACA GAG GCA CAG TTG AGT
431   R   Q   Y   M   F   S   S   L   K   N   N   K   K   C   T   P   T   E   A   Q   L   S
1357 GCT ATT GAT GAT CTG ATT GAG TCT ATG AGC TTG GTA AAG AAA AGC GAG GAA GAA GAT ACC ATT GAA
453   A   I   D   D   L   I   E   S   M   S   L   V   K   K   S   E   E   D   T   I   E
1423 GAC TTG TTT CCA ACC TCC AAA ATT CCA AAT CCC GAA TTT CAG AGA TTT TTC CAG TGT CTG CTG CAC
475   D   L   F   P   T   S   K   I   P   N   P   E   F   Q   R   F   F   Q   C   L   L   H
1489 AGA GTC TTA CAT CCC CAA GAG CGT TTG CCC CCA ATT CAG CAG CAC ATT TTG AAT ATG CTG AAT CTC
497   R   V   L   H   P   Q   E   R   L   P   P   I   Q   Q   H   I   L   N   M   L   N   L
1555 CCC ACT GAG ATG AAA GCC AAA TGT GAG ATT CCT CTC TCT AAA GTG AGG ACC CTT TTC CCG CTG ACA
519   P   T   E   M   K   A   K   C   E   I   P   L   S   K   V   R   T   L   F   P   L   T
1621 GAA GCC GTC AAG AAA AAG GAC CAA GTG ACT GCT CAG GAC ATT TTC CAA GAC ATT CAC GAA GAG GGG
541   E   A   V   K   K   K   D   Q   V   T   A   Q   D   I   F   Q   D   I   H   E   E   G
1687 CCC GCT GCC AAA AAA TGT AAG ACA GAG AAA GAA GAA GGT CAC ATC AGT ATC TCT AGC GTG GCT GAA
563   P   A   A   K   K   C   K   T   E   K   E   E   G   H   I   S   I   S   S   V   A   E
1753 GGG AAT GTC ACC AAG GTT GGA AGT GTG AAT CCT GTT GAA AGC TTC CGT GTT CTA GTG AGG CAG AAG
585   G   N   V   T   K   V   G   S   V   N   P   V   E   S   F   R   V   L   V   R   Q   K
1819 ATT GCC AGC TTT GAG CAG GCA AGT CTC CAG CTA ATA AGT CAC ATT GAG CAG TTT TTG GAT ACC AAC
607   I   A   S   F   E   Q   A   S   L   Q   L   I   S   H   I   E   Q   F   L   D   T   N
1885 GAA ACG CTG TAT TTT ATG AAG AGT ATG GAG TGC ATC AAA GCT TTC CGG GAG GAG GCC ATC CAG TTT
629   E   T   L   Y   F   M   K   S   M   E   C   I   K   A   F   R   E   E   A   I   Q   F
1951 TCA GAA GAG CAG CGC TTC AAC AGC TTC CTG GAA GCC CTT CGA GAG AAA GTG GAA ATT AAG CAA TTA
651   S   E   E   Q   R   F   N   S   F   L   E   A   L   R   E   K   V   E   I   K   Q   L
2017 AAT CAT TTC TGG GAA ATT GTT GTT CAG GAT GGA GTT ACT CTG ATT ACC AAG GAT GAA GGC TCT GGA
673   N   H   F   W   E   I   V   V   Q   D   G   V   T   L   I   T   K   D   E   G   S   G
2083 AGC TCT GTC ACA ACT GAG GAA GCC ACA AAG TTC CTG GCT CCC AAA GAC AAA GCA AAA GAA GAT GCA
695   S   S   V   T   T   E   E   A   T   K   F   L   A   P   K   D   K   A   K   E   D   A
2149 GCA GGA CTT GAA GAA GGT GGC GAT GTG GAT GAT TTA CTG GAT ATG ATA TAG acc atg gat gtg ttg
717   A   G   L   E   E   G   G   D   V   D   D   L   L   D   M   I   *
2215 gga atc taa gca tgc cat ctc cgt tgc tga gag ctc aga aag agc atc ctg gaa gcc at
```

FIG. 5. Nucleic acid and predicted amino acid sequences of Chinese hamster Ku86 cDNA. The deleted nucleotides in XR-V9B and XR-V15B are underlined. Lowercase letters, untranslated nucleotides; asterisk, stop codon.

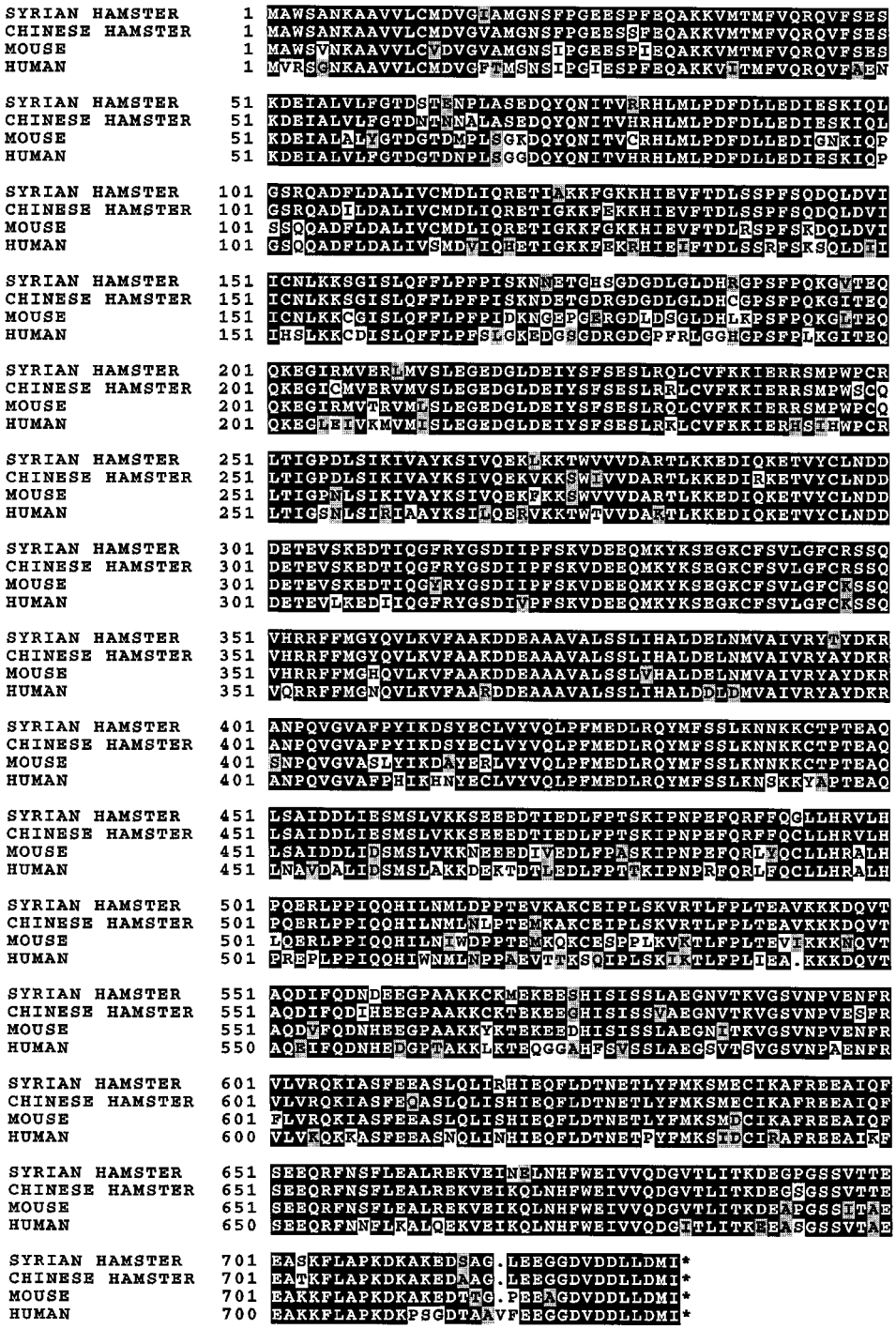

FIG. 6. Amino acid alignment of Syrian and Chinese hamster, mouse, and human Ku86 proteins. Alignment was determined by using the Boxshade program (8a). Identical amino acids are highlighted in black. Functionally conserved amino acids are highlighted in gray. Conserved amino acids are classified as follows: V, I, L, and M; D, E, Q, and N; F, Y, and W; G, S, T, P, and A; and K, R, and H.

the XR-V9B cDNA (Fig. 6). These deletions do not shift the reading frame and correspond to a deletion of 46 amino acid residues from codons 372 through 417 in XR-V15B and to a deletion of 84 codons from codons 267 through 350 in XR-V9B (Fig. 6). No additional mutations in these Ku86 cDNAs could be detected in either mutant cell line.

To eliminate the possibility that the deletions in XR-V15B and XR-V9B were cloning artifacts, RT-PCR was performed with primers adjacent to these deletions. Primers Ku-12 and Ku-13 were designed to generate a 448-bp fragment spanning the deletion in the XR-V15B mutant, and primers Ku-5 and Ku-8b were used to generate a 1,246-bp fragment spanning the deletion in XR-V9B. RT-PCR showed that the XR-V15B mutant contained a PCR fragment consistent with a length of 310

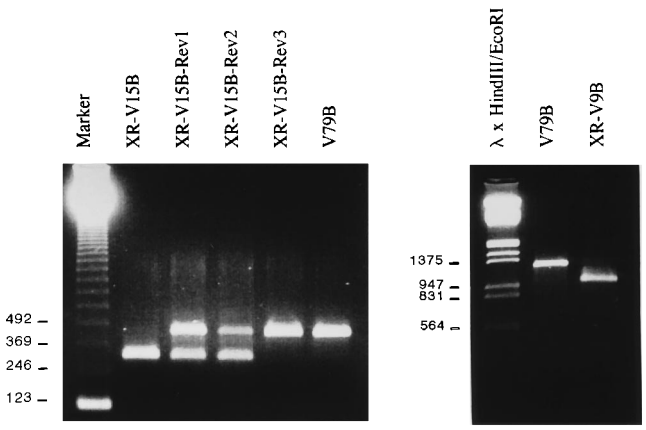

FIG. 7. RT-PCR of XR-V15B, XR-V15B revertants, and V79B with primers Ku-12 and Ku-13 (left) and of V79B and XR-V9B with primers Ku-5 and Ku-8b. The positions of molecular size markers (in base pairs) are shown on the left of each panel.

bp, instead of the wild-type fragment of 448 bp (Fig. 7). Similarly, XR-V9B contains a PCR fragment consistent with a length of 994 bp, instead of the wild-type 1,246-bp fragment (Fig. 7). Thus, we observed PCR products shorter than the wild-type product, confirming the deletions in the Ku86 cDNAs in both mutants.

RT-PCR analysis of X-ray-resistant revertants of XR-V15B. To examine the mechanism responsible for the reversion of the X-ray sensitivity in XR-V15B, we analyzed three X-ray-induced X-ray-resistant revertant cell clones, XR-V15B-Rev1, XR-V15B-Rev2, and XR-V15B-Rev3 (see Materials and Methods). All three revertants showed wild-type levels of X-ray resistance (data not shown), suggesting that Ku86 function was completely restored in each case. To determine the status of the deleted cDNA in these revertants, RT-PCR was performed with primers Ku-12 and Ku-13. Revertants XR-V15B-Rev1 and XR-V15B-Rev2 showed a normal, wild-type fragment of 448 bp, as well as a smaller fragment of 310 bp (Fig. 7). Thus, we concluded that the XR-V15B-Rev1 and XR-V15B-Rev2 cell lines expressed one mutant and one wild-type copy of Ku86. In contrast, XR-V15B-Rev3 expressed only a wild-type fragment of 448 bp (Fig. 7). This indicated that an event leading to the disappearance of the deletion occurred in XR-V15B-Rev3.

DISCUSSION

The data presented in this paper demonstrate that defects in the *Ku86* gene are responsible for the phenotypes of complementation group 5 hamster mutants. First, transfection of XR-V15B cells with a cDNA encoding the hamster *Ku86* gene was capable of fully restoring DEB activity, Ku70 protein levels, X-ray resistance, and V(D)J recombination. Second, XR-V15B and XR-V9B cells express mRNAs which encode Ku86 proteins containing significant in-frame deletions. This is the first direct evidence that mutant cells in group 5 contain mutations in the *Ku86* gene. Thus, the *XRCC5* gene defective in group 5 cells is identical to the *Ku86* gene.

Rescue of group 5 mutant cells with hamster Ku86 cDNA. The expression of Syrian hamster Ku86 cDNA in XR-V15B Chinese hamster mutant cells was capable of restoring DEB activity to levels comparable to those of the wild-type V79 cell line. The Ku70 protein levels in the clone expressing the highest level of DEB activity increased to wild-type levels (Fig. 1, lane 5). The restoration of Ku70 levels in mutant cells by hamster Ku86 suggests that a functional Ku86 protein is necessary for stabilization of the Ku70 polypeptide. Conversely, there is evidence that functional Ku70 protein is required for stabilization of Ku86 (39). Thus, it appears that if Ku70 and Ku86 are not complexed to each other, they are degraded in the cell.

Our results suggest that Ku levels are limiting in the processes of DNA repair and V(D)J recombination. Although clones expressing wild-type levels of DEB activity showed complete restoration of X-ray resistance and V(D)J recombination activity, clones expressing less than wild-type levels of DEB activity showed only partial rescue of X-ray resistance and V(D)J recombination. Between 16 and 40 DNA DSBs are induced in mammalian cells by 1 Gy of ionizing radiation (11). Therefore, even the highest dose used in our experiments, 6 Gy, induced at most 240 DSBs per cell. Each wild-type human cell contains about 400,000 molecules of Ku (30). Therefore, we were surprised to find that transfected cells expressing Ku at levels only modestly less than wild-type levels were partially impaired in both X-ray resistance and V(D)J recombination.

There are several possible explanations. Multiple Ku molecules might be necessary to process each end of a broken DNA molecule. Although Ku binds initially to DNA ends, it can translocate inward along the DNA molecule either in an ATP-independent manner or as an ATP-dependent helicase (9, 48). Whether these functions are required for the repair of DNA DSBs remains unknown. Alternatively, Ku might be localized to subcellular compartments that limit its access to DNA strand breaks. In fact, Ku has been reported to change nuclear localization in a cell cycle-dependent way (49). Additionally, Ku is known to be posttranslationally modified (5, 33); it is possible that only one form of Ku is able to function in DNA repair. Thus, in cells expressing low levels of Ku, the functional form of Ku may be present at levels too low to completely restore DNA repair. Finally, it is possible that Syrian hamster Ku86, whose amino acid sequence is 4.5% diverged from Chinese hamster Ku86, rescues Chinese hamster mutants less efficiently so that Ku levels only appear to be limiting. To rule out this possibility, we are currently transfecting the Chinese hamster Ku86 clone into Chinese hamster mutant cells.

Defects in the mRNA from mutant cells in group 5. The deletions in mRNAs recovered from XR-V15B and XR-V9B cells do not shift the open reading frame but instead remove 46 and 84 amino acids from two different regions within the encoded Ku86 protein. The deletion of either region leads to an apparent failure to stabilize Ku70 protein, the loss of DEB activity, and the concomitant impairment of X-ray resistance and V(D)J recombination in the cells. Therefore, the deleted domains appear to play an essential role in the function of the Ku86 protein.

Chinese hamster cell lines have been the source of a broad spectrum of phenotypically recessive mutants corresponding to a large number of different genetic loci (8, 41). Normally, recessive mutations should not be found at such high frequencies in diploid cells. Siminovitch (41) has suggested that a substantial part of the genome in Chinese hamster cell lines is effectively haploid so that many genes are represented by only one functional allele. Functional hemizygosity can arise from the silencing of genes by methylation, and group 5 hamster cell mutants might have two alleles of the *XRCC5* gene, with one allele inactivated by mutation and another wild-type allele silenced by methylation (22). RNAs derived from revertants XR-V15B-Rev1 and XR-V15B-Rev2 harbor both wild-type and deleted Ku86 sequences. Thus, a wild-type *Ku86* gene was activated in these revertants. Consistent with this observation,

we have previously shown that azacytidine, an agent which induces demethylation of silenced genes, greatly increases the frequency of revertants in XR-V15B (36, 37). Therefore, the mechanism for activating the *Ku86* gene in these revertants appears to be via demethylation of a silenced allele.

The third revertant, XR-V15B-Rev3, contained only wild-type Ku86 mRNA, suggesting that demethylation was not involved in reactivation of this gene. The deleted mRNA region may have reappeared in this revertant by several mechanisms: (i) homologous recombination between the active mutant allele and the inactive normal allele; (ii) gene conversion of the active allele, with the inactive allele as a template; or (iii) reversion of the mutation in the active (defective) allele. The third possibility suggests that the deletion in the Ku86 mRNA is the result of defective splicing caused by a revertible mutation at a donor or acceptor splice site in the gene. Significantly, the XR-V15B mutant was induced by ethylnitrosourea, which almost exclusively induces point mutations (40), so the molecular defect in XR-V15B might be a point mutation in the genomic DNA which leads to defective splicing of the Ku86 mRNA. In support of this possibility, the 3′ end of each deletion contains a short pyrimidine-rich tract followed by an AG dinucleotide, which is similar to the consensus mRNA splice acceptor sequence and thus could act as an acceptor for defective splicing (Fig. 5). However, confirmation of such mutations will require the sequencing of genomic DNA.

In summary, the rescue of XR-V15B cells by a Ku86 cDNA and the identification of defects in the cDNAs encoding Ku86 in XR-V15B and XR-V9B cells demonstrate that (i) the defect in complementation group 5 mutant cells must be in the *Ku86* gene, (ii) Ku86 is essential for DSB repair and V(D)J recombination, and (iii) Ku86 expression is required for the accumulation of Ku70 protein.

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A.E. and V.S. contributed equally to this work.

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