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Molecular and Biochemical Characterization of *xrs* Mutants Defective in Ku80

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The gene product defective in radiosensitive CHO mutants belonging to ionizing radiation complementation group 5, which includes the extensively studied *xrs* mutants, has recently been identified as Ku80, a subunit of the Ku protein and a component of DNA-dependent protein kinase (DNA-PK). Several group 5 mutants, including *xrs-5* and *-6*, lack double-stranded DNA end-binding and DNA-PK activities. In this study, we examined additional *xrs* mutants at the molecular and biochemical levels. All mutants examined have low or undetectable levels of Ku70 and Ku80 protein, end-binding, and DNA-PK activities. Only one mutant, *xrs-6*, has Ku80 transcript levels detectable by Northern hybridization, but Ku80 mRNA was detectable by reverse transcription-PCR in most other mutants. Two mutants, *xrs-4* and *-6*, have altered Ku80 transcripts resulting from mutational changes in the genomic Ku80 sequence affecting RNA splicing, indicating that the defects in these mutants lie in the Ku80 gene rather than a gene controlling its expression. Neither of these two mutants has detectable wild-type Ku80 transcript. Since the mutation in both *xrs-4* and *xrs-6* cells results in severely truncated Ku80 protein, both are likely candidates to be null mutants. Azacytidine-induced revertants of *xrs-4* and *-6* carried both wild-type and mutant transcripts. The results with these revertants strongly support our model proposed earlier, that CHO-K1 cells carry a copy of the Ku80 gene (*XRCC5*) silenced by hypermethylation. Site-directed mutagenesis studies indicate that previously proposed ATP-binding and phosphorylation sites are not required for Ku80 activity, whereas N-terminal deletions of more than the first seven amino acids result in severe loss of activities.

A DNA double-strand break (DSB) potentially represents a catastrophic lesion to a cell, threatening the integrity of its genome. DNA DSBs can be induced directly by a variety of damaging agents, most notably ionizing radiation. They can also arise indirectly during the processing of other forms of DNA damage and may even occur as a result of endogenous damage arising from the cells' own metabolism. The processes of V(D)J recombination in vertebrates and mating-type switching in yeast cells involve the introduction of transient endonuclease-induced DSBs (for reviews, see references 1 and 17). Also, meiotic recombination, an essential step in successful meiosis, necessitates the introduction and recombinational repair of multiple DSBs. It is not surprising, therefore, to find that all cell types have invested resources into repairing such lesions.

Three complementation groups of rodent cell lines, designated ionizing radiation (IR) groups 4, 5, and 7, have been identified as having defects in DSB repair (30, 49, 50). The members of these complementation groups have similar phenotypes, including, as predicted, pronounced radiosensitivity (54). Recent advances have resulted in the cloning or identification of the genes defined by these complementation groups, namely, *XRCC4*, *-5*, and *-7*. *XRCC4* encodes a ubiquitously expressed protein which has no identifiable domains or homology to other proteins (36). The products of *XRCC5* and *-7* are components of the same protein complex, namely, DNA-dependent protein kinase (DNA-PK) (3, 31, 39, 44, 48). DNA-PK consists of (i) a heterodimeric protein (Ku) which serves to target the complex to double-stranded DNA (dsDNA) ends, and (ii) a large catalytic subunit (DNA-PKcs) (12, 19). *XRCC5* encodes Ku80, the larger subunit of the Ku protein (44, 48), and *XRCC7* encodes DNA-PKcs (3, 31, 37, 39). Mutants defective in Ku70 have not yet been identified but would be predicted to be radiosensitive. Significantly, the mutants belonging to IR groups 4, 5, and 7, in addition to showing pronounced radiosensitivity, harbor defects in the ability to undergo V(D)J recombination (38, 46, 47). Indeed, IR group 7 includes the *scid* cell line, derived from the SCID mouse, identified on the basis of a severe immune defect and subsequently shown to be radiosensitive and defective in DSB rejoining (2, 4, 16, 22). These results, therefore, show that DNA-PK plays an essential role in both DNA DSB repair and V(D)J recombination, and they demonstrate a mechanistic overlap between these two processes.

Among the first radiosensitive rodent mutants to be described were a group, designated *xrs-1* to *xrs-7*, isolated in a single mutant hunt using the CHO-K1 cell line (28). Cell fusion studies established that all except *xrs-3* belonged to a single complementation group, subsequently designated IR group 5 (24, 50). These mutants represented the first mammalian mutants exhibiting a defect in DNA DSB rejoining and have been used extensively in many laboratories (especially *xrs-5* and *-6*) to validate and optimize techniques for measuring DNA DSB rejoining, to examine the consequences of defective rejoining, and to investigate the mechanism of the rejoining process in mammalian cells (partly reviewed in reference 25; see also references 9, 10, and 23). More recently, other mutants belonging to this group have also been isolated (13, 35, 55). Although the six *xrs* mutants share the same overall phenotype of a major sensitivity to ionizing radiation and defect in DNA DSB rejoining, the finer details of their properties differ. For example, the pattern of cross-sensitivity to DNA-damaging

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TABLE 1. Primers used for PCR and site-directed mutagenesis

Name	Sequence	Position
AP34	5′AAA GTA ACC AAA CCG CCC GTG GAC3′	−30 to −7
AP24	5′ATT TTG GAG GTT GGA AAC AAG TCT T3′	1445 to 1421
AP35	5′GAG TGG TCA TTC TGT AGA AAG TTG GG3′	3′ untranslated region
AP36	5′ATA TCA AGT TCT GAA GGT CTT TGC AG3′	1074 to 1099
AP48	5′CCC GTG GAC CAG CAA CAT GG3′	−16 to +4
C	5′ATG TGC AGC TGC CTT TCA TGG AAG3′	1262 to 1285
X6I3	5′TTG CTT GTT CAA ACG AGG ATT CTT3′	103 to 80
X6I2	5′CAG AAA ACA CCT GAC GTT3′	145 to 128
X6I1	5′GCC CGT GGA CCA GCA ACA T3′	−17 to +2
X614	5′ACC CAT AGC AAC GCC CAC ATC3′	63 to 43
217	5′TGG ATG CAA GAA CTC TAA AGA3′	836 to 856
218	5′TCA TTT GTT CCT CAT CCA CTT3′	994 to 974
X4D3	5′AAG TGG ATG AGG AAC AAA TGA3′	974 to 994
X4D10	5′CTT CAG CAC TTG ATA TCC CAT AAA3′	1088 to 1065
SDM5	5′ACA GCA CAA CAG CCA TGT TGC TGG T3′	37 to −9
SDM6	5′GCT GCT CTT CTG CAA ACT GGA TGG C3′	1963 to 1939
SDM7	5′GAG CCT TCA TCC CGG GTA ATC AGA GT3′	2078 to 2053
SDM11	5′CCT GTC GTT GGA CCA TGT TGC TGG TC3′	136 to −10
SDM12	5′GAT ACT GGT CCT CCA TGT TGC TGG TC3′	223 to −10

agents differs between the mutants (26, 28). Properties associated with the defects in Ku have been characterized for *xrs-5* and/or *xrs-6*. The defective phenotypes of both mutants can be complemented by human Ku80 cDNA, *xrs-5* and *-6* have no detectable Ku-dependent dsDNA end-binding activity, and *xrs-6* cells have also been shown to lack DNA-PK activity, a result predicted from the defect in Ku and the requirement in vitro for Ku to activate the kinase catalytic component of DNA-PK (8, 14, 15, 18, 40, 44, 48).

To gain further insight into the nature of the defect in Ku80, we have examined additional *xrs* mutants which have not hitherto been analyzed for these biochemical defects. We also report on the defects in Ku80 at the molecular level and consider the results in light of the differing properties of the mutants. Another intriguing feature of the *xrs* mutants is their ability to revert at high frequency following treatment with azacytidine, a powerful demethylating agent (27). A "silent gene" hypothesis was proposed to explain these results, in which it was suggested that the parent CHO-K1 cell line contained an inactive methylated copy of the *xrs* gene (*XRCC5*). We present results which provide support for this model. We also report the results of a deletion analysis of the Ku80 protein.

MATERIALS AND METHODS

Cell culture and DNA transfections. The *xrs* cell lines were derived from the CHO-K1 cell line on the basis of their sensitivity to ionizing radiation (28). Cells were cultured in minimal essential medium (Gibco) supplemented with nonessential amino acids, penicillin, streptomycin, glutamine, and 10% fetal calf serum as described previously. Transfection was carried out by using the Polybrene transfection method as described previously (29). Transfectants were selected by using 600 μg of G418 per ml and subsequently maintained in 300 μg of G418 per ml. Determination of survival after ionizing radiation and PCR analysis of transfectants were done as described previously (20).

DNA and RNA extraction. DNA was extracted by standard procedures. Poly(A)$^+$ RNA was extracted from 5×10^7 cells, using a Quickprep Micro mRNA Purification kit (Pharmacia Ltd.).

cDNA library screening. A CHO cDNA library was kindly supplied by I. Hickson and screened by standard procedures, using a final wash of 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 65°C.

cDNA synthesis and sequencing. Reverse transcription (RT)-PCR was carried out with 1 to 5 μg of poly(A)$^+$ RNA, oligo(dT) primers, and reverse transcriptase, followed by amplification using primers AP34 and AP24 for the 5′ half of the gene and primers AP36 and AP35 for the 3′ half of the gene, using procedures described earlier (6). The products were purified by electrophoresis through low-melting-point agarose, and small amounts were used in a second-round PCR amplification step performed with (i) biotinylated AP48 and nonbiotinylated AP24 or (ii) biotinylated AP35 and nonbiotinylated C. The biotinylated PCR products were extracted by using streptavidin-coated magnetic beads and sequenced directly by using a variety of primers (not shown) and a Thermo Sequence Cycle sequencing kit (Amersham Life Science Inc.). To sequence products containing intron sequences and to verify the sequencing of specific regions, amplified products obtained by using nonbiotinylated primers were cloned into a T vector (33) and sequenced from dsDNA. In this case, a minimum of four clones derived from each product were sequenced to distinguish PCR errors from mutational changes.

PCR. PCR was carried out in 20 μl containing 2 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 15 mM $MgCl_2$, 0.1% gelatin), 1.5 μl of deoxynucleoside triphosphates (2.5 mM each dATP, dCTP, dGTP, and dTTP), 2 to 5 pmol of each oligonucleotide primer, and 0.25 U of *Taq* polymerase (HT Biotechnology Ltd.). Primers used in this study are listed in Table 1.

Northern blot analysis. Two to 3 μg of poly(A)$^+$ RNA was size separated by electrophoresis in 1% agarose-formaldehyde gels, transferred overnight to Hybond N (Amersham) in 20× SSC, baked at 80°C for 2 h, and UV cross-linked. The probe was a hamster Ku80 cDNA clone missing the first 600 bp of the open reading frame (ORF) and labeled by random priming. Hybridization was carried out under standard conditions.

End-binding and DNA-PK assays. DNA end-binding assays were carried out essentially as described previously (19, 48). In brief, extracts were prepared by a modification of the method of Scholer et al. (42) and were incubated with γ-^{32}P-labeled double-stranded oligonucleotide M1/M2 at room temperature for 30 min; DNA-protein complexes were resolved on 4% polyacrylamide gels containing 5% glycerol. DNA-PK assays were carried out essentially as previously described (14).

Immunoblotting. Whole-cell extracts (120 μg) were boiled in SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer and separated by SDS-PAGE (8% gel). Proteins were transferred to nitrocellulose by using a wet-blotting apparatus and blocked for 1 h at room temperature or overnight at 4°C with 2% skimmed milk solution and 0.05% Tween. The primary antibodies were Ku80-4 and Ku70-5, which were raised against baculovirus-expressed Ku80 and Ku70 proteins, respectively (Serotech, Oxford, United Kingdom). Primary antibody (Ku80-4 or Ku70-5 diluted 1/5,000 or 1/2,500, respectively, in 2% milk solution) was added for 1 to 2 h at room temperature and washed extensively in milk solution, and anti-rabbit immunoglobulin antibody (diluted 1/2,500) was added for 1 h at room temperature. The filter was rewashed and developed with an ECL kit (Amersham).

Construction of hamster Ku80 cDNA in pcDNA3. The hamster Ku80 cDNA was amplified by RT-PCR using primers AP34 and AP35 and cloned into a T vector (33), and the 5′ region was recovered by digestion with *Eco*RI and *Pfl*MI, which cut within the multicloning site of the vector and at position 731 of the Ku80 cDNA, respectively. A partial Ku80 cDNA clone obtained from screening a hamster library was cloned into the *Xho*I site of pcDNA3 (Invitrogen); the incomplete 5′ region of this clone was removed by digestion with *Eco*RI and *Pfl*MI and replaced with the PCR-amplified 5′ fragment to generate a complete hamster cDNA construct. A single PCR error in the PCR-amplified portion of the gene was corrected by site-directed mutagenesis.

Site-directed mutagenesis of hamster Ku80 cDNA. SDM 5, 6, 7, 11, and 12 were constructed by the Kunkel method of site-directed mutagenesis (34), using the oligonucleotides listed in Table 1. These constructs therefore use the endog-

enous ATG site. SDM 343 was constructed by removing an *Eco*RI/*Pfl*MI fragment from pcDNA3 containing wild-type Ku80 and replacing it with a PCR-amplified fragment starting at the ATG site at bp 343. This construct therefore utilizes the ATG at bp 343 as its initiation codon. The entire Ku80 cDNA was sequenced in all clones after construction to verify that no other sequence alterations had occurred during the construction.

RESULTS

Biochemical characterization of the *xrs* mutants. Previous studies have shown that IR group 5 mutants are defective in Ku80, a subunit of the Ku protein (5, 44, 48). A key feature in the identification of this defect was the observation that *xrs-5* and *xrs-6* cells lack dsDNA end-binding activity (15, 18, 40, 48). The known role of the Ku protein as the DNA end-binding component of DNA-PK led to the prediction that group 5 mutants might also be defective in DNA-PK activity, and this was verified in assays using *xrs-6* cells (14). We have extended these studies to examine three additional *xrs* mutants for these biochemical endpoints.

(i) Examination of dsDNA end-binding and DNA-PK activities. *xrs-1*, *-4*, *-5*, *-6*, and *-7* cells all showed a complete lack of DNA end-binding activity (Fig. 1A) and low or negligible DNA-PK activity (Fig. 1B). We estimate that the limit of detection of dsDNA end-binding activity is 5% of the level present in parental cells. We are unable to determine whether the low residual kinase activity seen in all *xrs* mutants represents residual DNA-PK activity or contaminating kinase activity eluting in the pull-down purification step, and we do not attach any significance to it.

(ii) Examination of Ku80 and Ku70 protein levels in *xrs* mutants by immunoblotting. To examine whether residual protein levels could be detected in the *xrs* mutants, the mutants were examined by Western immunoblotting using polyclonal antibody Ku80-4, which is an anti-human Ku80 antibody that cross-reacts with the hamster Ku80 protein. No material cross-reacting with antibody Ku80-4 was detected in the extracts derived from any of the *xrs* mutants (Fig. 2A). As observed previously, each subunit of the Ku protein is required to stabilize the other, and the absence of Ku80 in the group 5 mutants has been shown to result in loss of the Ku70 component (5, 8, 44, 48). To examine levels of the Ku70 subunit in the mutants, immunoblotting was carried out with antibody Ku70-5, an anti-human Ku70 antibody that cross-reacts well with the rodent protein. All *xrs* mutants show dramatically decreased levels of Ku70 protein (Fig. 2B). A very low residual level of material cross-reacting with antibody Ku70-5 was detectable in extracts from all mutants.

Expression of Ku80 transcript in *xrs* mutants. To assess whether Ku80 mRNA was expressed in the *xrs* mutants, poly(A)$^+$ mRNA was isolated from them and from parental K1 cells and examined by Northern hybridization using a partial hamster Ku80 cDNA clone as a probe (see below) (Fig. 3). In assays using 2 to 3 μg of poly(A)$^+$ mRNA, only *xrs-6* cells contained detectable levels of Ku80 transcript, with levels similar to that observed in parental cells.

As a more sensitive method to detect low transcript levels, RT-PCR was carried out on the *xrs* mutants, and all gave detectable amplification products, showing that at least a low level of Ku80 transcript was present in all mutants.

Sequence of Chinese hamster Ku80 cDNA. To examine the *xrs* mutants for the presence of mutations in the Ku80 gene, it was first necessary to derive the parental Chinese hamster Ku80 sequence. PCR-amplified human Ku80 cDNA was used as a probe to screen a Chinese hamster cDNA library under low-stringency conditions. The largest cDNA clone obtained had a 1.2-kb insert but was lacking 600 bp from the 5′-terminal

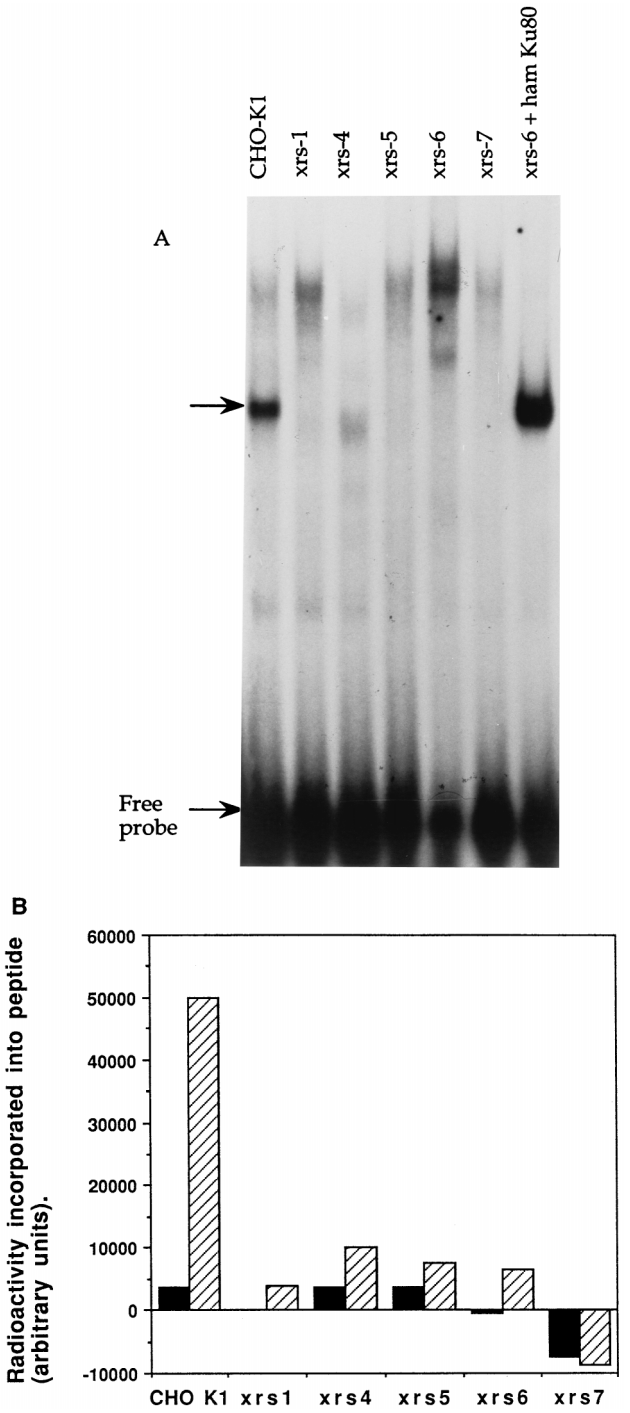

FIG. 1. (A) Absence of DNA end-binding activity in *xrs* cells. Whole-cell extracts from CHO-K1 cells, *xrs* mutants, and an *xrs-6* transfectant containing hamster Ku80 cDNA were mixed with a radiolabeled double-stranded oligonucleotide probe, and DNA-protein complexes were separated by PAGE as described in Materials and Methods. The faint band in the *xrs-4* sample was not routinely observed and is different in size from the Ku-dependent band. (B) Absence of DNA-PK activity in *xrs* cells. The pull-down DNA-PK assay was used to measure DNA-PK activity from whole-cell extracts of CHO-K1 cells and *xrs* mutants. Assays were carried out in the presence of wild-type p53 peptide (striped bars) that is recognized by DNA-PK as well as other kinases and in the presence of a mutated peptide (black bars) which is not an effective kinase substrate. We consider that the levels observed in all *xrs* mutants are within the experimental variability of background.

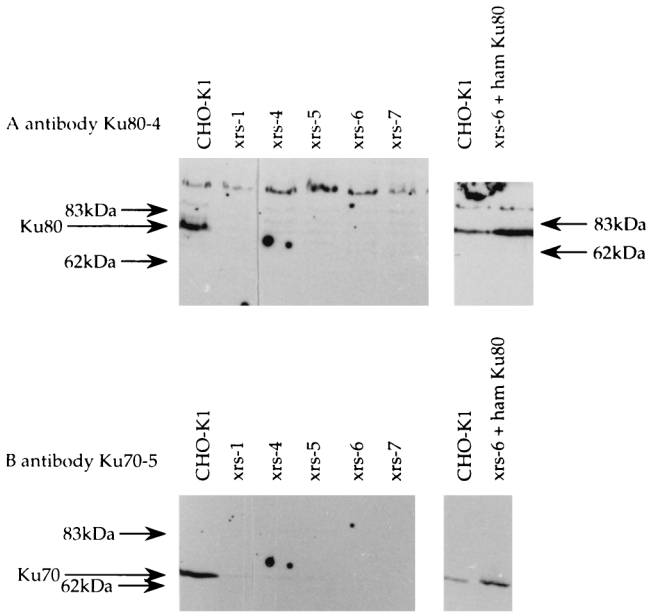

FIG. 2. Immunoblot analysis of *xrs* cells. Whole-cell extracts from CHO-K1 cells, *xrs* mutants, and an *xrs-6* transfectant containing hamster (ham) Ku80 cDNA were examined by Western blotting using antibody Ku80-4 (A) and antibody Ku70-5 (B).

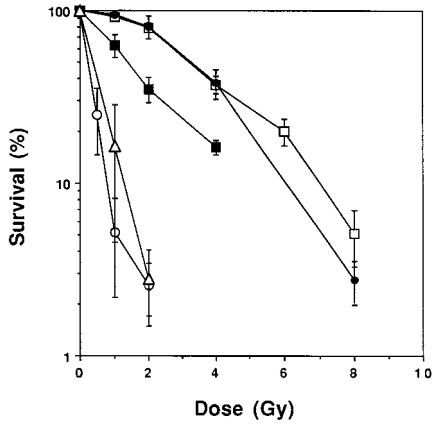

FIG. 4. Survival of CHO-K1 cells, *xrs-6* cells, and an *xrs-6* transfectant containing hamster Ku80 cDNA following gamma irradiation. ●, CHO-K1; ○, *xrs-6*; ■, *xrs-6* transfectant carrying human Ku80 cDNA; □, *xrs-6* transfectant carrying hamster Ku80 cDNA; △, *xrs-6* transfectant carrying *xrs-6* mutation.

portion of the gene. This region was obtained by RT-PCR from CHO-K1 cells, using primers for the 5′ region derived from the mouse Ku80 sequence and a reverse primer derived from the incomplete hamster Ku80 cDNA described above. The entire Ku80 hamster sequence was examined by RT-PCR using CHO-K1 cells followed by direct sequencing and by sequencing the region covered by the hamster cDNA clone. Our sequence is similar to a Chinese hamster Ku80 sequence recently published (13) (GenBank accession number L48606), with two differences at bp 168 and 698 (position 1 is the A at the start of the ORF). In both cases, A is present in place of G; the former is a silent change, but the latter results in a glutamine instead of an arginine in the protein sequence.

Complementation of *xrs-6* cells. Previous studies have shown that the human Ku80 cDNA can significantly, but not fully, complement the defects in radiosensitivity, DSB rejoining, and V(D)J recombination characteristic of *xrs-6* cells (44, 48). To examine whether the hamster Ku80 cDNA could fully restore radioresistance to the *xrs-6* cells, the entire hamster Ku80 ORF was cloned into the mammalian expression vector pcDNA3 (see Materials and Methods). pcDNA3 containing the intact hamster Ku80 ORF (pcDNA3hamKu80) was transfected into *xrs-6* cells, and transfectants were selected by using the neomycin resistance marker present in this plasmid. Control transfections were also carried out with vector alone. Individual clones were examined for the presence of the Ku80 cDNA by PCR using primers AP34 and AP35, which amplify the Ku80 ORF. Clones positive by this criterion were examined for radiosensitivity. Full restoration of gamma-ray resistance was obtained in all clones examined (Fig. 4). A single clone was also examined for DNA end-binding activity, and full restoration to parental levels was observed (Fig. 1A). Ku80 and Ku70 protein levels were examined by immunoblotting, and levels slightly greater than those found in the CHO-K1 parent were observed (Fig. 2). These data are compatible with similar results obtained in assays using other members of IR group 5 as recipients (13, 21) and contrast with the partial correction obtained in assays using human Ku80 cDNA (5, 44, 48).

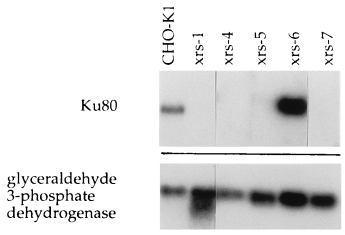

FIG. 3. Northern analysis of Ku80 from *xrs* cells. In panel A, the probe was a hamster Ku80 cDNA fragment missing the 5′ portion of the cDNA; in panel B, the probe was glyceraldehyde 3-phosphate dehydrogenase. Northern analysis was carried out as described in Materials and Methods.

Sequence analysis of Ku80 cDNAs from the *xrs* mutants. To sequence Ku80 cDNA from the mutants, RT-PCR was carried out as described in Materials and Methods followed by direct sequencing. The results obtained with each mutant are described below.

(i) *xrs-1*. The level of Ku80 mRNA was very low as detected by Northern blot analysis, and we were unable to amplify Ku80 cDNA reproducibly from *xrs-1* even from cDNA preparations from which other cDNAs could be successfully amplified. We were able to sequence 2.0 kb of the 2.2-kb ORF from the successful experiments, and no mutations were detected. We therefore conclude that the sensitivity of *xrs-1* results from low transcript levels.

(ii) *xrs-4*. RT-PCR sequence analysis of Ku80 mRNA from *xrs-4* cells revealed a 245-bp deletion from positions 801 to 1045. Additionally, RT-PCR using primers flanking the deletion confirmed the presence of the deletion and provided evidence for two different-size products (Fig. 5) both smaller than that from normal cells. This was in contrast to the results obtained by sequence analysis, where only a single smaller sequence was detected. A likely explanation, however, is that PCR may selectively amplify one transcript relative to the other so that neither the sequence nor PCR analysis provides an accurate assessment of the relative levels of these transcripts within the cell. These results suggested that one, or possibly two, exons had been deleted from the transcript in *xrs-4* cells and that the causal mutation might lie in a site affecting RNA splicing. We predicted that the region deleted

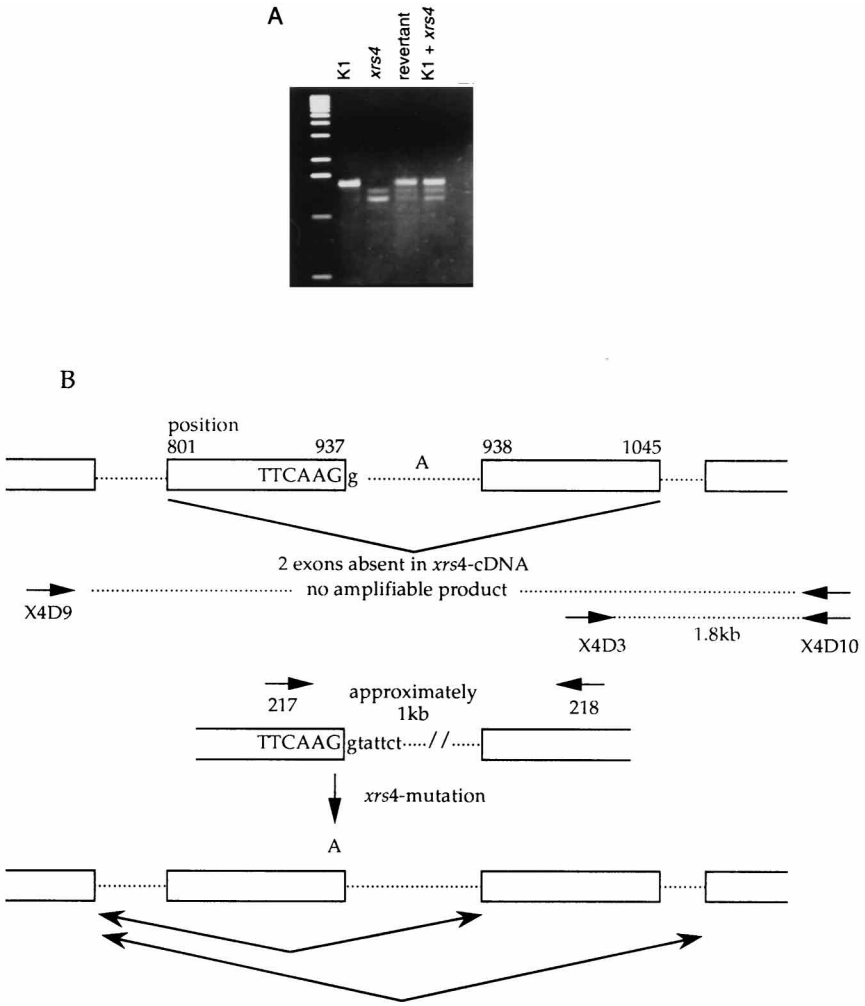

FIG. 5. Detection of the mutation in *xrs-4* cells. (A) Identification of 245-bp deletion in cDNA of *xrs-4* cells. RT-PCR was carried out with CHO-K1 cells, *xrs-4* cells, an azacytidine-induced revertant of *xrs-4* cells, and equal quantities of CHO-K1 and *xrs-4* mRNAs, using primers AP34 and AP24. The products were separated through 2% agarose gels. (B) Identification of the mutation in genomic DNA of *xrs-4* cells. Primers 217 and 218 were used to amplify genomic DNA from CHO-K1 and *xrs-4* cells. The 1-kb PCR product was cloned and sequenced (see Materials and Methods). A mutational change from G to A was observed in two of five clones derived from *xrs-4* cells. Both of the two clones derived from CHO-K1 cells gave the sequence expected from the cDNA sequencing. The intron sequence obtained was identical in all clones derived from CHO-K1 and *xrs-4* cells. The figure depicts the likely splicing products obtained in *xrs-4* cells. Only the product missing two exons was detected by sequencing following RT-PCR.

in the transcript would be present in genomic DNA and would be flanked by introns. To examine the splice sites associated with these intron/exon boundaries, we attempted to amplify this region from genomic DNA. Primer pairs X4D3-X4D10 and X4D9-X4D10 were used to amplify genomic DNA (Fig. 5); the former gave a 1.8-kb product, whereas we were unable to detect an amplified product with the latter primers. Using primers 217 and 218, we obtained a PCR product of approximately 1 kb, compared with a size of 158 bp expected from cDNA. This result indicates the presence of a further intron (A in Fig. 5) which we located between bp 937 and 938. Since intron A lies within the 245 bp deleted in *xrs-4* cDNA, this result demonstrates that two exons were deleted in the *xrs-4* transcript. Sequence analysis of the product amplified with primers 217 and 218 identified a G-to-A mutation in the exon base pair immediately preceding intron A in approximately half of the clones derived from *xrs-4* cells. The scoring system of Shapiro and Senapathy (43) for likelihood of usage of splice sites gives a rather low score of 70 for the splice donor sequence of intron A. The G-to-A mutation in *xrs-4* reduces this score to 58, which is probably too low for this to be used effectively as a splice donor site. Using additional primers (not shown), we also amplified the intron between bp 800 and 801 from CH0-K1 and *xrs-4* cells and found identical sequences in the intron/exon boundary regions in both lines. We were unfortunately unable to clone the intron between bp 1045 and 1046. From these results, we conclude that two copies of *XRCC5* are present in *xrs-4* cells. In one of these copies, we have identified a mutation affecting splicing of Ku80 mRNA, which is most likely the causal mutation in *xrs-4* cells, although we have not excluded the possibility of a second mutation in the downstream flanking intron/exon boundary. The 245-bp deletion caused by the loss of two exons gives rise to a shift in the reading frame, with a stop codon encountered 61 bp downstream of the deletion. The result will be a truncated protein of 287 amino acid residues.

(iii) *xrs-6*. We identified a 13-bp insertion at sequence position 21 in the transcript present in *xrs-6* cells. To verify this,

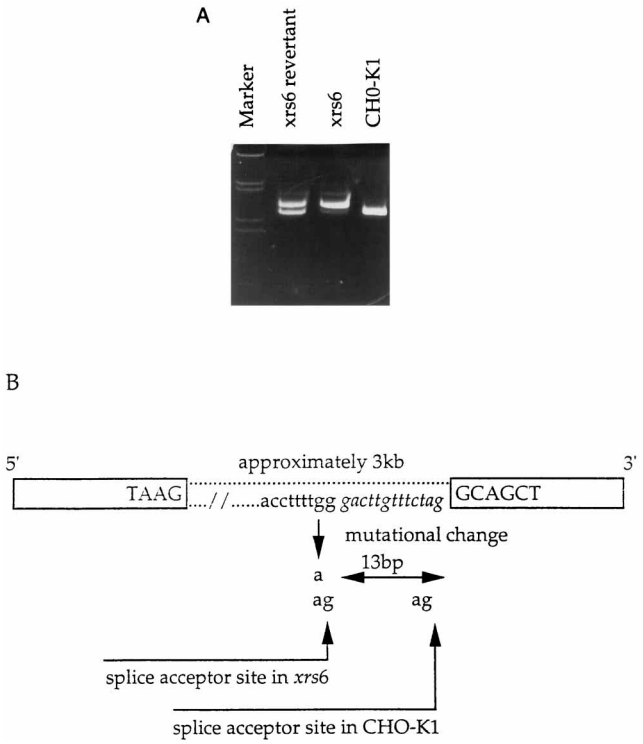

FIG. 6. Detection of the mutation in *xrs-6* cells. (A) Identification of a 13-bp insertion in cDNA of *xrs-6* cells. RT-PCR was carried out with CHO-K1 cells, *xrs-6* cells, and an azacytidine-induced revertant of *xrs-6* cells, using primers X6I1 and X6I2, and the products were separated through 12% polyacrylamide gels. Sequence analysis revealed the 13-bp insertion shown in panel B. (B) Identification of the mutation in genomic DNA of *xrs-6* cells. Primers AP34 and X6I3 were used to amplify genomic DNA from *xrs-6* and CHO-K1 cells. AP34 and X6I4 were used in a second-round PCR, and the 3-kb product obtained was cloned and partially sequenced (see Materials and Methods). The results showed that the inserted 13 bp were derived from the 3′ end of the intervening intron. A mutational change (G to A) in the intron sequence in *xrs-6* cells was observed in half (four of eight) of the clones derived from *xrs-6* cells. Both of the two clones from CHO-K1 cells gave the wild-type sequence shown. Sequences in uppercase represent exon sequences, those in lowercase are intron sequences, and those in italics are the intron sequences present in *xrs-6* cDNA. The site of the mutational change is as indicated. The mutational change creates a new ag splice acceptor site as shown.

RT-PCR was carried out with primers which flanked this region (Fig. 6B), and the amplified products were separated by gel electrophoresis (Fig. 6A). The results confirmed the presence of a larger product from *xrs-6* cells compared with CHO-K1 cells. We anticipated that the mutation might lie in a site affecting RNA splicing and that the inserted bases might be derived from the genomic sequence. To examine this, we used primers flanking the insertion and were able to amplify across an intervening intron from genomic DNA of CHO-K1 and *xrs-6* cells (Fig. 6). Sequence analysis showed that the inserted sequences were derived from the 3′ (splice acceptor) end of the intervening intron and identified a mutational change from G to A in approximately half of the clones derived from *xrs-6* cells at a site two bases preceding the inserted sequence. This mutational change results in the creation of a new AG splice acceptor sequence 13 bp upstream of the normal splice site and the insertion of the first 13 bp of the intron into the following exon. As in the case of the *xrs-4* cells, the presence of this mutational change in approximately half of the clones derived from *xrs-6* cells suggests that two copies of the *XRCC5* gene are present in the *xrs-6* cells and that only one copy carries the identified mutation. This insertion results in a frameshift. A stop codon is present 41 bp downstream of the insertion, so that a truncated protein of only 25 amino acid residues will be produced, and we therefore considered that this represented the likely causal mutation in *xrs-6* cells. To verify this, we constructed the 13-bp insertion in the Ku80 cDNA in pcDNA3 and introduced this into *xrs-6* cells by DNA transfection. Neomycin-resistant transfectants were selected, and individual clones, shown to carry the intact Ku ORF by PCR, were examined for their ability to complement the radiosensitivity of *xrs-6* cells. We were unable to detect any change in survival levels of five clones analyzed, and results for one of these clones are shown in Fig. 4. These results verify our conclusion that this is the causal mutation in *xrs-6* cells.

(iv) *xrs-5* and *xrs-7* cells. The levels of Ku80 transcripts were very low as judged by Northern analysis. We were, however, able to identify and sequence RT-PCR products from both types of cells, and the sequences obtained were identical to that found in CHO-K1 cells. We therefore conclude that the sensitivity of these cells results from the low transcript levels.

Analysis of azacytidine-induced revertants. In previous work (27), we have shown that the *xrs* mutants revert at high frequency following treatment with azacytidine. As an explanation for these results, we postulated that the parent CHO-K1 cells contained two copies of *XRCC5*, one of which was inactivated by methylation. We argued that the ethyl methanesulfonate mutagenesis used to isolate the *xrs* mutants had resulted in mutations in the remaining active copy while retaining the silent but intact allele. Azacytidine treatment causes activation of this silent allele, a phenomenon described as epimutagenesis. Identification of the mutational defect in *xrs-4* and *xrs-6* cells therefore allows us to test the predictions of this hypothesis. First, analysis of genomic DNA of *xrs-4* and *xrs-6* cells showed two copies of the gene, only one of which was mutated, as expected if one copy is silent. Second, revertants of *xrs-4* and *xrs-6* cells were isolated following azacytidine treatment, amplified by RT-PCR, and examined by direct sequencing. The direct sequencing method that we use allows the identification of two alleles with a single base pair difference as a double sequence at the site in question. Deletions in one allele are seen as a stretch of double sequences. Revertants derived from *xrs-4* and *xrs-6* both contained double sequences at the predicted sites.

To gain further evidence for the presence of both parental and mutant cDNAs in *xrs-4* and *xrs-6* revertants, they and the relevant parental strains were amplified by RT-PCR using primer pairs X6I1-X6I2, and AP34-AP24 respectively. The *xrs-6* revertant clone contained both the larger products seen in *xrs-6* cells and smaller product present in CHO-K1 cells, indicating the presence of both wild-type and mutant cDNAs (Fig. 6A). As described above, the 245-bp deletion in *xrs-4* cells results in two major PCR products, both smaller than that present in CHO-K1 cells. The *xrs-4* revertants regain the parental PCR product while retaining the smaller products (Fig. 5A). The ratio of the two alternatively spliced forms may not represent the relative frequency of these two cDNAs within the cell due to differences in their amplification rates. However, it is noteworthy that the wild-type band in the revertants is considerably stronger than that in either mutant product. This result may indicate that the abnormally spliced product in the *xrs-4* mutant is unstable and is consistent with our inability to see any detectable Ku80 transcript in Northern analysis of *xrs-4* poly(A)$^+$ RNA.

Taken together, the results for these two mutants suggest that treatment with azacytidine results in the reappearance of the wild-type transcript together with the continued presence

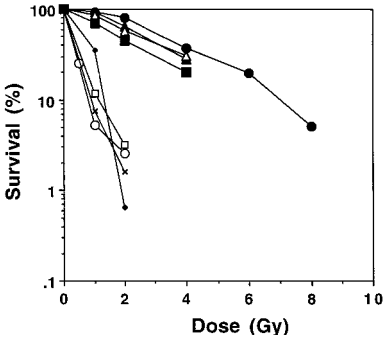

FIG. 7. Survival of *xrs-6* cells containing mutant hamster Ku80 cDNA following gamma irradiation. ●, CHO-K1; ○, *xrs-6*; ■, *xrs-6* transfected with SDM 7; □, *xrs-6* transfected with SDM 343; △, *xrs-6* transfected with SDM 6; ▲, *xrs-6* transfected with SDM 5; ×, *xrs-6* transfected with SDM 11; ◆, *xrs-6* transfected with SDM 12.

of the mutant transcript. These are the results predicted by the epimutagenic model for azacytidine-induced reversion.

Site-directed mutagenesis of Ku80. To gain further insight into the function of Ku80, we have commenced a structure-function analysis of the Ku80 gene. We have constructed a series of N-terminal deletions in the hamster Ku80 cDNA in pcDNA3 as described in Materials and Methods and introduced them into *xrs-6* cells by DNA transfection. Neomycin-resistant transfectants were selected, and individual clones, shown to carry the truncated Ku80 cDNA by PCR, were examined for the ability to complement the radiation sensitivity of *xrs-6* cells, for the presence of Ku80 and Ku70 proteins by Western blot analysis, and for DNA end-binding activity (Fig. 7 and 8; summarized in Table 2). The functions of Ku80 were largely unaffected by deletion of the first 21 bp. In contrast, all larger 5′ deletions failed to correct the radiosensitivity of *xrs-6* cells or to restore end-binding activity even though the Ku80 subunit could, in all cases, be detected by Western blot analysis. However, the truncated proteins appeared unable to stabilize and, by implication, to interact with Ku70, since no appreciable elevation of Ku70 levels were detected above the severely reduced levels observed with *xrs-6* cell extracts (Fig. 2 and 8). The higher apparent levels of Ku70 compared with Ku80 in the lines carrying the wild-type hamster protein are most likely due to the enhanced ability of the human Ku70 antibody used to cross-react with the hamster Ku70 protein compared with the Ku80 antibodies, rather than representing actual differences in protein levels.

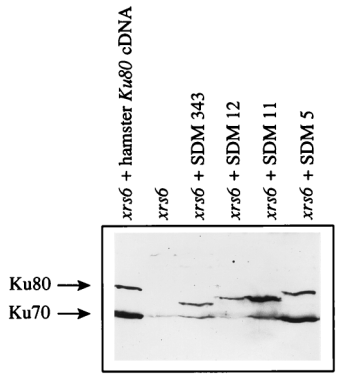

FIG. 8. Immunoblot analysis of *xrs-6* cells containing N-terminal deletions in the hamster Ku80 cDNA. Whole-cell extracts from *xrs-6* cells containing the intact hamster Ku80 cDNA and N-terminal deletions were examined by Western blotting using antibody Ku80-4 together with antibody Ku70-5.

We also created mutations in two sites which have been suggested to be potentially functional domains. Cao et al. (7) reported that Ku might possess DNA-ATPase activity and identified a motif claimed to bear homology to a conserved sequence motif of DNA-ATPases identified by Koonin (32). To address whether this motif might be important for function, we mutated the lysine residue (amino acid position 689) to arginine and examined the mutant cDNA for its ability to complement *xrs-6* cells. Good complementation of radiosensitivity was observed, suggesting that this sequence is not essential for Ku's function in DSB repair. Ku80 has also been reported to be phosphorylated at a serine residue in vivo and in vitro, and the latter can be carried out by DNA-PK (52, 53), although a DNA-PK recognition sequence has not been identified in the protein. The serine residue at amino acid position 651 was suggested to be a possible phosphorylation site, and therefore we mutated this site to alanine. This mutated protein was also able to complement *xrs-6* cells efficiently, suggesting that if phosphorylation does occur at this site, it is not important for function.

DISCUSSION

In this study, we have characterized the biochemical and molecular defects in five of the *xrs* mutants belonging to IR complementation group 5. The molecular basis for the sensitivity of *xrs-2*, the sixth member, is currently under investigation, and the results of this work will be reported elsewhere. Previous studies have characterized the biochemical properties of some members of this complementation group and have shown that they can be attributed to a defect in the Ku80 component of the Ku protein (14, 15, 18, 40, 44, 48). We show here that the five *xrs* mutants have major defects in dsDNA-end binding and DNA-PK activities. Using the human anti-Ku80 and anti-Ku70 antibodies, we found that all of the *xrs* mutants had severely reduced levels of Ku80 and Ku70 proteins. Since the two components of the Ku protein stabilize each other, another aspect of the group 5 phenotype is decreased levels of the Ku70 component of the Ku protein (5, 8, 48). However, very low residual levels of material cross-reacting with both antibodies could be detected in all mutants, which may indicate low residual Ku80 protein levels in all mutants or could be due to other proteins cross-reacting weakly with the antibodies. The Ku80-4 antibody gave other cross-reacting bands of different sizes, but the Ku70-5 antibody gave only a band of 70 kDa. The mutants were examined with a second anti-human Ku70 antibody (N3H10), and again a very low residual level of cross-reacting material was observed.

Ku80 transcript is undetectable in four of the five *xrs* mutants by Northern analysis, although the presence of low levels of transcript is indicated by our ability to amplify their mRNAs by using the more sensitive technique of RT-PCR. Sequencing of the RT-PCR products from two of these three mutants (*xrs-5* and *xrs-7*) failed to identify any mutational change. We therefore conclude that the phenotype of three of the *xrs* cell lines, *xrs-5*, *xrs-7*, and *xrs-1* (from which no RT-PCR product could be reproducibly amplified), results from low expression of Ku80 mRNA. The molecular basis for this will require sequencing of the entire Ku80 gene and/or regulatory sequences. Mutational changes in the Ku80 gene were, however, detected in two mutants (*xrs-4* and *-6*). In *xrs-6* cells, a mutational change in an intron sequence creates a new splice site 13 bp upstream of the original, unaltered splice site. As expected from rules governing splicing, in which the first AG down-

TABLE 2. Analysis of clones derived from *xrs-6* cells transfected with mutant Ku80 cDNA

Clone	Mutation	*xrs-6* radiosensitivity	Ku80 protein level	Ku70 protein level	DNA end-binding activity
SDM 5	5′ 21 bp deleted	Resistant	Wild type	Wild type	Wild type
SDM 11	5′ 120 bp deleted	Sensitive	Wild type	Low (*xrs-6* level)	Undetectable
SDM 12	5′ 207 bp deleted	Sensitive	Reduced	Low (*xrs-6* level)	Undetectable
SDM 343	5′ 342 bp deleted	Sensitive	Reduced	Low (*xrs-6* level)	Undetectable
SDM 6	Amino acid residue 689	Resistant	Wild type	Wild type	Wild type
SDM 7	Amino acid residue 651	Resistant	Wild type	Wild type	Wild type

stream of the branch site is used as the splice acceptor, splicing at this new site appears to dominate and results in the majority of the cDNA being out of frame and the generation of a translation product of only 24 amino acid residues. We have shown that Ku80 cDNA carrying the 13-bp insertion is unable to rescue the radiosensitivity of *xrs-6* cells, verifying its complete loss of function.

We have identified a 245-bp deletion in the cDNA of *xrs-4* cells, representing two deleted exons, and a mutational change in one copy of the genomic DNA that destroys a splice donor site at the central exon/intron boundary. Although it might not be anticipated that a mutation in this splice site would result in loss of the adjacent 5′ and 3′ exons, similar effects have been reported for splice site mutations in other genes (e.g., see reference 45). The very low mutant transcript levels in *xrs-4* cells indicate additionally that this aberrant splicing also causes the transcripts to be unstable, a feature which has been observed previously for nonsense mutations (41). The deletion causes much of the remaining cDNA sequence to be out of frame and will result in a truncated protein of 287 amino acid residues. Whether *xrs-4* and *xrs-6* represent null mutants will be discussed in more detail below. These results, at least for *xrs-4* and *-6*, verify that the defect lies in the Ku80 gene itself rather than in a gene controlling its expression. Two other group 5 mutants, XR-V15B and XR-V9B have also been shown to carry deletions in Ku80 cDNA, although the mutational change in genomic DNA was not ascertained (13).

An important question in assessing the phenotype of these cells is whether they represent null mutants or whether residual Ku is present. Residual DNA end-binding activity could not be detected in any mutant. Residual kinase activity was detectable in our DNA-PK assay, but we are unable to determine whether this represents residual DNA-PK or a contaminating kinase activity. Thus, these assays have limited sensitivity. RT-PCR of two mutants (*xrs-4* and *-6*) failed to demonstrate the presence of any wild-type transcript. Since both mutations result in truncated products, which in the case of *xrs-6* results in only the very N-terminal portion of the protein being expressed, *xrs-4* and *-6* are likely candidates to represent null mutants. The other mutants examined (*xrs-1*, *-5*, and *-7*) yielded low levels of wild-type Ku80 transcripts, suggesting that they are not completely null for Ku80. However, these mutants have as marked a phenotype as *xrs-4* and *-6*, and indeed *xrs-5* displays the largest defect in DSB rejoining. In addressing explanations for these low transcript levels, the ability of all *xrs* mutants to revert should be considered (27). Although reversion occurs at high frequency following azacytidine treatment, most workers using these cells, including ourselves, have experienced the occasional spontaneous reversion of *xrs* populations. The low level of wild-type Ku80 transcript present in some *xrs* cells could, therefore, represent the presence of a few revertants in the *xrs* populations or could represent a low wild-type transcript level present in each cell. Although great care was taken to verify the radiosensitivity of the cell population from which the RNA was made, our survival assay would not detect a low frequency of revertant cells, whereas they might be detectable in the RT-PCR amplification step. It is also possible that transient undermethylation immediately following replication could result in transient expression of a wild-type transcript from the silent allele in the replicated but not yet methylated gene (see reference 11 for a discussion on this speculation). Since *xrs-4* and *-6* cells also revert following azacytidine treatment, these explanations would predict that a low level of wild-type transcript might be present in these cells. Although our results do not exclude this possibility, no evidence for the presence of a wild-type transcript was obtained. Alternative explanations for low wild-type transcript levels in the mutants include mutations in Ku80 regulatory sequences, mutations resulting in decreased mRNA stability, or leaky mutations affecting RNA splicing. The phenotypes of cell lines derived from knockout Ku80 mice, which are currently being constructed by several laboratories, will ultimately provide a means to examine the consequences of complete loss of Ku80 protein.

Our analysis of revertants of *xrs-4* and *-6* cells provides strong support for the notion that these cells harbor an intact but nonexpressed Ku80 allele, which can be reactivated by treatment with azacytidine. The tendency of these cells to revert spontaneously suggests that such spontaneous reactivation can occur at low frequency without exposure to azacytidine.

We also report our initial observations aimed at elucidating a structure-function relationship for Ku80. Our results show that the first 21 bp of the cDNA are dispensable for Ku's function in DSB repair, whereas loss of the 5′ 120 bp affects the ability of the Ku80 subunit to interact with Ku70. Attempts to express Ku80 protein in vitro have indicated that Ku80 is unstable in the absence of Ku70 and vice versa, a feature supported by our observations in vivo that Ku70 levels are decreased in *xrs* mutants. Therefore, the ability to detect Ku80 but not Ku70 protein (Fig. 8) was surprising and suggests that the truncated Ku80 proteins are stable when not complexed to Ku70, unlike the wild-type protein, due either to protein conformational changes or to loss of degradation signal sequences. These results indicate that the N-terminal region of Ku80 may be required for efficient Ku70-Ku80 interaction. This could be because (i) this region contains a functional interaction domain, (ii) it affects some other function of the protein, such as transport to the nucleus, which precludes subunit interaction, or (iii) it is required to maintain the three-dimensional structure of the protein such that interaction can take place. We (23a) and others (51) have observed that other regions of Ku80 are also important for its interaction with the 70-kDa subunit, but our results reported here are not incompatible with those observations. We have also shown that two sites, previously proposed as potential candidates for ATP binding and phosphorylation, are not important for Ku function.

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