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Ku86 Is Not Required for Protection of Signal Ends or for Formation of Nonstandard V(D)J Recombination Products

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Ku, a heterodimer of 70- and 86-kDa subunits, serves as the DNA binding component of the DNA-dependent protein kinase (DNA-PK). Cells deficient for the 86-kDa subunit of Ku (Ku86-deficient cells) lack Ku DNA end-binding activity and are severely defective for formation of the standard V(D)J recombination products, i.e., signal and coding joints. It has been widely hypothesized that Ku is required for protection of broken DNA ends generated during V(D)J recombination. Here we report the first analysis of V(D)J recombination intermediates in a Ku-deficient cell line. We find that full-length, ligatable signal ends are abundant in these cells. These data show that Ku86 is not required for the protection or stabilization of signal ends, suggesting that other proteins may perform this function. The presence of high levels of signal ends in Ku-deficient cells prompted us to investigate whether these ends could participate in joining reactions. We show that nonstandard V(D)J recombination products (hybrid joints), which involve joining a signal end to a coding end, form with similar efficiencies in Ku-deficient and wild-type fibroblasts. These data support the surprising conclusion that Ku is not required for some types of V(D)J joining events. We propose a novel RAG-mediated joining mechanism, analogous to disintegration reactions performed by retroviral integrases, to explain how formation of hybrid joints can bypass the requirement for Ku and DNA-PK.

V(D)J recombination assembles the variable regions of immunoglobulin and T-cell receptor (TCR) genes during lymphocyte development (reviewed in reference 4). The recombination machinery recognizes sequence elements, termed recombination signal sequences (RSS), located adjacent to the V, D, and J coding elements. Recombination is initiated by introduction of double-strand breaks (DSB) precisely between the RSS and the coding sequence, generating two types of broken DNA ends: blunt, 5′-phosphorylated signal ends, which terminate in RSS, and covalently closed (hairpin) coding ends (42, 43, 45, 48, 59). These reaction intermediates join to form two standard products: signal joints, which typically retain all nucleotides of the RSS, and coding joints, which characteristically exhibit loss and/or addition of nucleotides.

Whereas cleavage is carried out by two lymphocyte-specific proteins, RAG-1 and RAG-2 (31), joining of the broken ends is performed with the participation of DNA repair activities expressed in many cell types. Several of these proteins were identified by analysis of mutations that affect both V(D)J recombination and DSB repair. For example, mice homozygous for the *scid* defect exhibit severely impaired formation of coding joints (28, 30) as well as hypersensitivity to agents that cause DSB (2, 11, 15). Additional mutant cell lines with defects in both V(D)J recombination and DSB repair have been isolated and assigned to four complementation groups, termed XRCC4 through XRCC7 (18, 19).

The *XRCC5* and *XRCC7* genes encode members of the DNA-dependent protein kinase (DNA-PK) complex. DNA-PK consists of a catalytic subunit ($DNA\text{-}PK_{CS}$ [encoded by the *XRCC7* gene]) and a DNA-binding component called Ku, a heterodimer of 70- and 86-kDa polypeptides (3, 21). The protein kinase activity of DNA-PK is activated when Ku binds to altered DNA structures, such as DSB, nicks, and hairpins (13, 33, 35, 37). Although the precise roles of DNA-PK in DSB repair and V(D)J recombination remain unknown, it has been proposed that DNA-PK might promote end-joining reactions, perhaps acting to align DNA termini or to recruit joining activities (3, 17).

The *XRCC5* gene encodes the 86-kDa subunit of Ku (referred to as Ku86) (54), and *xrcc5* mutant cell lines, such as xrs-6, are hypersensitive to ionizing radiation, fail to exhibit detectable Ku end-binding activity, and are generally severely defective for formation of both coding and signal joints (12, 38, 39, 53, 55). While no nucleotide sequences of coding joints isolated from *xrcc5* mutant cells have been reported, analysis of signal joints isolated from xrs-6 cells transfected with plasmid V(D)J recombination substrates revealed loss of nucleotides (38, 55). This is distinctly unusual, as signal joints from normal cells very rarely exhibit loss of even a single nucleotide (28, 29). Based on these observations, we and others have suggested that Ku86 may play a crucial role in protecting both coding and signal ends from nucleolytic degradation (3, 10, 12, 17, 39, 41, 54, 55).

The recent generation of Ku86-deficient mice in two different laboratories has allowed further characterization of the role of Ku86 in V(D)J recombination. Both studies found that formation of signal and coding joints is severely defective in Ku86-deficient mice (36, 58), confirming previous results obtained with xrs-6 cells. The two groups analyzed V(D)J recombination intermediates at different endogenous antigen receptor loci in thymocytes of Ku86-deficient mice, with divergent results. In one study, both initial products of the cleavage reaction, hairpin coding ends and intact signal ends, were detected at the TCRδ and -β loci (58). These data suggest that Ku86 is not required for protection of signal or coding ends. However, in the other study signal ends at the immunoglobulin heavy-chain locus were not detected, leading the authors to conclude that Ku is required for protection of signal ends (36). It is not clear whether the dissimilarities between these results

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are due to the examination of different loci, to different mutant *xrcc5* alleles, or to some other factor. However, since these experiments involve analysis of events which occur during lymphocyte differentiation, the interpretation of the results could be complicated by unanticipated effects of Ku deficiency on thymocyte differentiation or on the accessibility of particular loci for recombination (see Discussion).

To clarify this important issue and to further investigate the role of Ku86 in processing of V(D)J recombination intermediates, we undertook a molecular analysis of V(D)J recombination intermediates and products generated from extrachromosomal substrates in xrs-6 cells. We recently described detection of signal end recombination intermediates in fibroblasts cotransfected with plasmid recombination substrates and expression vectors encoding RAG-1 and RAG-2 (51). In this study we used the same system to demonstrate that while Ku86 is necessary for the formation of signal joints, it is not required for the protection or stabilization of signal ends. These data are consistent with a model in which broken DNA intermediates remain sequestered in a synaptic complex (presumably along with the RAG proteins) which cannot proceed to the joining step in the absence of Ku86.

Unexpectedly, we found that nonstandard V(D)J recombination products, termed hybrid joints (which involve joining a coding end to a signal end), form efficiently in *xrcc5* mutant fibroblasts. Furthermore, the majority of hybrid joints from both wild-type and *xrcc5* mutant fibroblasts formed without loss of nucleotides from either end, indicating that Ku86 is not needed for protection of these termini. These data demonstrate that joining of a signal end to a coding end can proceed efficiently under conditions which impair joining of these same ends to form coding and signal joints. We propose two different models to account for the ability of hybrid joints to bypass the requirements for Ku and DNA-PK.

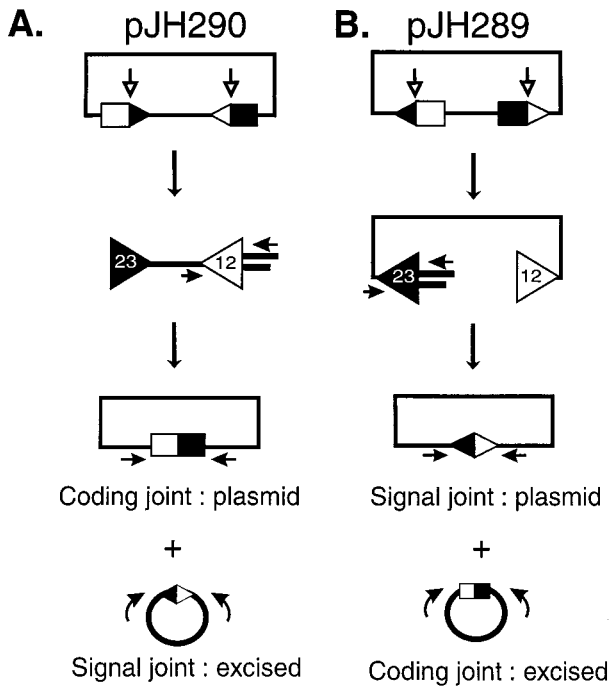


FIG. 1. Extrachromosomal V(D)J recombination substrates, expected intermediates, and products. DSB are introduced precisely between the RSS (triangles) and the coding segments (squares). Cleavage of pJH290 (A) produces signal ends on an excised molecule, whereas cleavage of pJH289 (B) produces signal ends on the plasmid. The joining of the signal ends yields signal joints on the excised circular molecules or on the plasmid. The two different recombination signals are symbolized as open (12-spacer) or filled (23-spacer) triangles. The vertical arrows represent the sites of cleavage. The filled arrows and a pair of heavy lines represent PCR primers and a double-stranded oligonucleotide for ligation, respectively.

MATERIALS AND METHODS

Cell culture. The cell lines CHOK1 4364 (20) (generously provided by T. Stamato) (referred to as CHOK1 hereafter in this work), xrs-6 (18) (generously provided by P. Jeggo, G. Taccioli, and F. Alt), and sc3T3 were maintained in Dulbecco's modified Eagle's medium enriched with 10% fetal bovine serum and incubated at 37°C in a humidified chamber containing a 5% CO_2 atmosphere.

Transfections. Transfections were done by a calcium phosphate method (CellPhect transfection kit; Pharmacia) as previously described (46, 51). Aliquots (2 to 5 μg) of the recombination substrates, pJH290, pJH289, and pJH299 (16, 26, 28), and truncated RAG-1 (pMS127b)/RAG-2 (pMS216) expression vectors (46, 47) were cotransfected transiently into cells. After 48 h, DNA was recovered as described previously (51). In each experiment, transfection efficiencies were determined by an established bacterial transformation assay which identifies DNA that has been replicated in eukaryotic cells by its resistance to digestion with *Dpn*I (38). Transfection efficiencies were similar in all three cell lines.

Analysis of signal joints and ends. To detect signal joints, DNA samples were subjected to PCR using DR55 and ML68 primers (51). Signal ends were detected by ligation-mediated PCR (LMPCR) as described previously (51, 59), using 24 cycles of amplification. The PCR primers for the 12-signal and 23-signal ends on the plasmid were DR99 and DR100, respectively. For the 12- and 23-signal ends on the excised product, DR55 and ML68 were used, respectively. The PCR primer for the ligated oligonucleotide (DR20) has been described previously (45). *Apa*LI digestion and T4 DNA polymerase treatment were performed as described previously (45, 59). PCR products were separated on 6% polyacrylamide gels, electrophoretically transferred to GeneScreen Plus membranes, and probed with an end-labeled oligonucleotide probe (DR73 or DR69) or an internally labeled *Pvu*II fragment of pJH290 which spans the region that undergoes rearrangement (51).

Assay for hybrid joints. Hybrid joints were amplified by PCR (24 cycles) using DR55 and ML68 primers. Amplified products were detected using an oligonucleotide probe (DR98) that hybridizes to the junction of coding and signal ends or to non-junction-specific probes such as DR55. PCR products containing hybrid joints were cloned by using the TA cloning kit (Invitrogen). Colonies containing hybrid joints were identified by colony hybridization (using radiolabeled, non-junction-specific oligonucleotide probes) or by PCR colony screening. Sequencing was performed with a Sequenase PCR product sequencing kit (U.S. Biochemicals).

Oligonucleotides. The following oligonucleotides were used in this study: DR73 (5′ GGTCGTTGATCCCCCATCGATGAGA 3′), DR98 (5′ ATGAGA GGATCCCACAGTGCTACA 3′), DR99 (5′ TCACACAGGAAACAGCTAT GACCATG 3′), and DR100 (5′ GGGATATATCAACGGTGGTATATCCA GTG 3′).

RESULTS

Experimental design. The extrachromosomal substrates used in this study and the expected intermediates and products of V(D)J recombination are diagrammed in Fig. 1. Recombination generates two reciprocal products: junctions retained on the plasmid (plasmid products) and reciprocal junctions formed on an excised circular molecule (excised products). Two different substrates were used to facilitate detection of both plasmid and excised recombination intermediates. Cleavage of pJH289 produces signal ends on the plasmid, whereas cleavage of pJH290 generates signal ends on an excised linear molecule (Fig. 1).

Substrates were introduced into mutant or wild-type CHOK1 cells, along with expression vectors encoding truncated versions of RAG-1 and RAG-2, as described previously (46, 51). DNA was recovered after 48 h, and signal ends were detected by a semiquantitative LMPCR (51, 59), as illustrated in Fig. 1. Products of V(D)J recombination were also detected by semiquantitative PCR amplification, as illustrated in Fig. 1. Amplified products were analyzed by polyacrylamide gel electrophoresis followed by hybridization to a radiolabeled probe. All transfections were repeated at least three times with similar results.

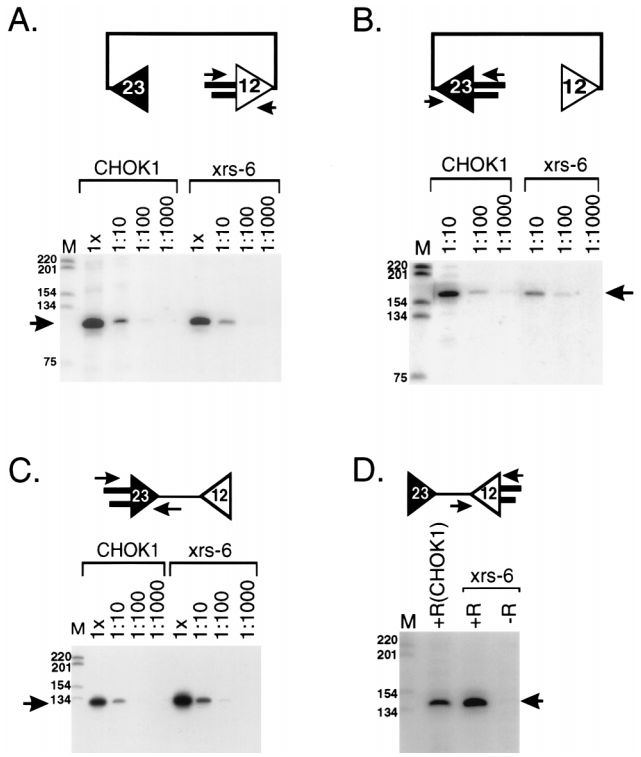


FIG. 2. Detection of signal ends by LMPCR. Blunt signal ends were detected by LMPCR. The amount of DNA added to each ligation reaction is indicated above each lane. 1× corresponds to 1/50 of the DNA recovered from each transfection. 1:10, 1:100, and 1:1,000 represent further dilutions. +R and −R signify the presence and absence, respectively, of the RAG-1 and RAG-2 expression vectors in the transfection. Lane M contains a radiolabeled 1-kb ladder (GIBCO BRL) (marker positions are indicated to the left of each gel). Blots were probed with the radiolabeled oligonucleotide DR69. Similar results were obtained with other probes, such as a random-primed *Pvu*II fragment of pJH290 (data not shown). (A) 12-spacer signal ends derived from cleavage of pJH289; (B) 23-spacer signal ends derived from pJH289 substrate; (C) 23-spacer signal ends on the excised circular molecule from pJH290; (D) 12-spacer signal ends derived from pJH290.

Full-length signal ends in xrs-6 cells. Analysis of signal ends derived from cleavage at the RSS containing the 12-nucleotide spacer (12-signal) of pJH289 is shown in Fig. 2A. Dilutions of DNA recovered from both cell lines generated amounts of LMPCR products that were proportional to the quantity of DNA tested. Limiting-dilution experiments showed that similar levels of signal ends were present in both wild-type and xrs-6 cells, as LMPCR products were detected in undiluted and 1:10 dilutions of transfected samples from both cell lines, trace amounts of products were seen in 1:100 dilutions, and no products were detected in 1:1,000 dilutions. Similar levels of signal ends were also detected in both cell lines by using PCR primers that amplify the 23-signal end (Fig. 2B). Thus, signal ends derived from cleavage of pJH289 are readily detected in xrs-6 cells, which do not express functional Ku86 and are severely defective for formation of signal joints (see below). Moreover, the signal ends are blunt and ligatable, since detection by LMPCR requires ligation to a flush, nonphosphorylated double-stranded oligonucleotide pair.

To examine signal ends on the excised fragment, we used the pJH290 substrate. As we observed with pJH289, 23-signal ends were detected in both wild-type CHOK1 and xrs-6 cells (Fig. 2C). Similar results were obtained in assays for the 12-signal end (Fig. 2D). As expected, signal ends were not observed when substrates were introduced into fibroblasts in the absence of RAG expression vectors (Fig. 2D), confirming that the LMPCR products detected in these assays are derived from RAG-mediated RSS cleavage. Thus, examination of cleavage products on both the plasmid and the excised fragments shows that blunt signal ends are present in xrs-6 cells at levels similar to those in wild-type cells.

The results described above indicate that the signal ends do not undergo substantial degradation in the absence of Ku86. Additional studies were performed to investigate the structure of the signal ends in more detail. Since ligation of the primers to a full-length signal end generates a novel *Apa*LI site (Fig. 3A), the integrity of signal ends can be assessed by digesting the LMPCR products with this restriction enzyme (51, 59). Analysis of 23- and 12-signal ends derived from cleavage of pJH290 from both wild-type and xrs-6 cells revealed virtually complete digestion with *Apa*LI (Fig. 3B). Thus, most blunt signal ends generated in both wild-type and Ku86-deficient cells retain all nucleotides of the RSS.

To search for signal ends that might terminate in short single-stranded extensions resulting from loss of nucleotides from either 5′ or 3′ termini, DNA samples were treated with T4 DNA polymerase prior to LMPCR to convert potential nonblunt ends to blunt ends (59). T4 DNA polymerase pre-

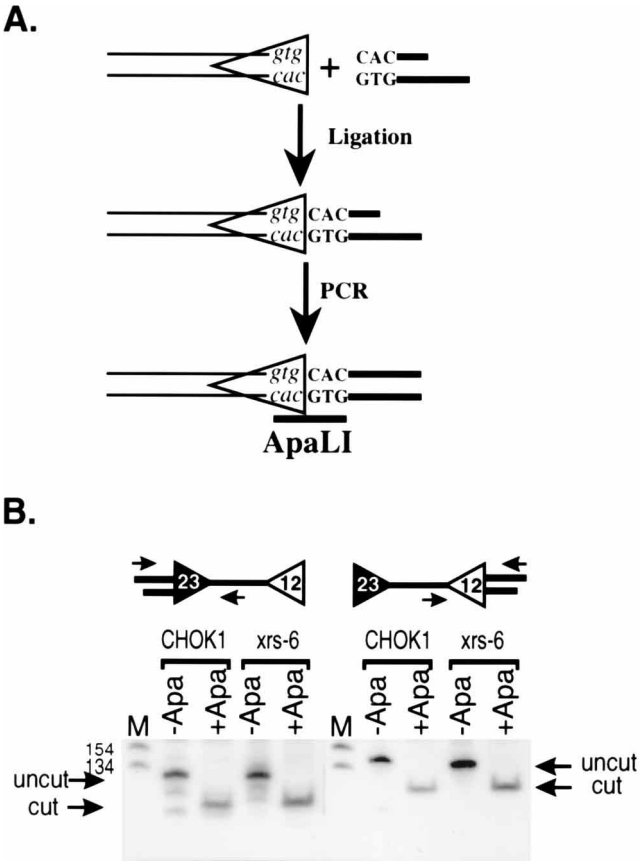


FIG. 3. Analysis of the structure of signal ends. (A) Schematic diagram of the *Apa*LI digestion assay. A full-length, blunt signal end generates an *Apa*LI restriction site upon ligation with the double-stranded oligonucleotide pair DR19-DR20. Signal ends with deletion or addition of nucleotides would not be cut by *Apa*LI. (B) LMPCR products derived from the 12- or 23-signal end were subjected to digestion with *Apa*LI. LMPCR from uncut and cut samples were loaded in adjacent lanes. Blots were probed with a random-primed *Pvu*II fragment from pJH290. Numbers to the left of the gel are marker (M) sizes (in kilobases).

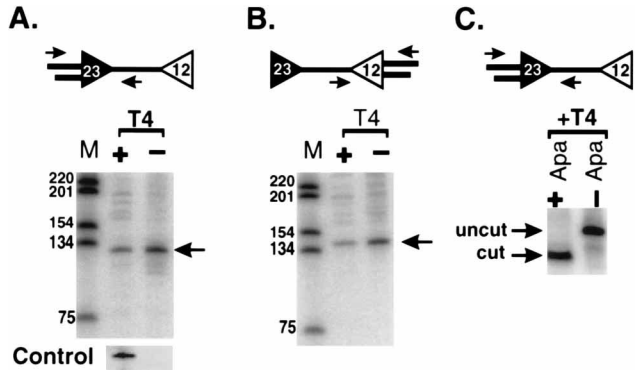


FIG. 4. Nonblunt signal ends are not detected in xrs-6 cells. To detect nonblunt signal ends, DNA samples were treated with T4 DNA polymerase prior to ligation. + and −, presence or absence, respectively, of T4 DNA polymerase in the reactions. (A) 23-spacer signal ends from pJH290. As a control for the activity of T4 DNA polymerase, BALB/c testis DNA was digested with *Pst*I restriction enzyme, which produces 3′ extensions. (B) 12-spacer signal ends from pJH290. (C) *Apa*LI restriction digestion of T4 DNA polymerase-treated DNA. Blots were probed with a random-primed *Pvu*II fragment from pJH290. Similar results were obtained for signal ends on the pJH289 plasmid (data not shown). Numbers to the left of the gels in panels A and B are marker (M) sizes (in base pairs).

treatment did not result in increased levels of LMPCR products (Fig. 4A and B), indicating that significant levels of nonblunt signal ends were not present. Control samples containing 3′ extensions treated in parallel verified that the T4 polymerase treatments were effective (Fig. 4A).

To search for nonblunt ends in an even more sensitive fashion, LMPCR products derived from T4 DNA polymerase-treated 23-signal ends were challenged with *Apa*LI. Virtually all the PCR products were *Apa*LI sensitive, indicating that the 3′ exonuclease activity of T4 polymerase did not convert potential 3′ single-stranded extensions to blunt ends (Fig. 4C). Similar results were observed for the signal ends derived from pJH289 (data not shown). These data demonstrate that the majority of signal ends generated by RAG-mediated cleavage in xrs-6 cells are blunt and full length, indicating that Ku86 is not required to preserve the integrity of signal ends after cleavage.

Since we detected abundant, ligatable signal ends in both wild-type and xrs-6 cells, we wanted to confirm that joining of these ends to form signal joints was indeed defective in the mutant cells used in our experiments. Therefore, a sensitive PCR that employs the same primers used to detect signal ends was used to assay for the corresponding signal joints. Whereas PCR products derived from signal joints on excised products derived from pJH290 were readily observed in wild-type cells (even at a 1:1,000 dilution), no products of the appropriate size were detected in the same DNA preparations from xrs-6 cells that contained abundant signal ends, even when large amounts of DNA were tested (Fig. 5). Similar results were obtained by a PCR designed to detect signal joints retained on the plasmid (data not shown). These results are in agreement with previous studies which examined plasmid products by using a different assay (53, 55). Thus, whereas signal ends are readily detected in both CHOK1 and xrs-6 cells, the mutant cells are severely defective for formation of signal joints.

Abundant hybrid joints in *xrcc5* and *xrcc7* mutant cells. Since the experiments described above demonstrate that abundant, ligatable signal ends are present in Ku86-deficient cells, we wondered whether these ends might be available for some joining reactions. Specifically, we searched for nonstandard V(D)J recombination products termed hybrid joints, which result from joining a coding end to a signal end (25, 26). To assay for formation of hybrid joints we used the pJH299 substrate (Fig. 6A), which forms inversions (standard product) and, about three- to sevenfold less frequently in wild-type cells, hybrid joints (26). Two hybrid joints can be generated from this substrate: a plasmid product and an excised product (Fig. 6A).

Excised hybrid joints derived from pJH299 were detected by semiquantitative PCR amplification in xrs-6 and wild-type CHOK1 fibroblasts. We also examined *scid* sc3T3 (*xrcc7* mutant) fibroblasts, which are defective for coding joint formation. The PCR products from all three cell lines were the expected size and hybridized to oligonucleotide probes specific for hybrid joints (Fig. 6B) as well as to non-junction-specific probes (data not shown), indicating that they are derived from authentic hybrids. The identity of these products was confirmed by nucleotide sequence analysis (see below).

Two approaches were used to determine the effects of *xrcc5* and *xrcc7* mutations on the efficiency of hybrid joint formation. First, levels of PCR products derived from hybrids were compared with levels of signal ends in each cell line (Fig. 6B). In all three cell lines, levels of hybrid joints correlated well with the abundance of signal ends. These data indicate that the *xrcc5* and *xrcc7* mutations do not significantly alter the efficiency of hybrid joint formation. Second, levels of PCR products derived from hybrids and inversions were compared. Limiting dilution analysis indicated that in wild-type cells the abundance of inversions was roughly 10-fold higher than the abundance of hybrids (data not shown), which is in agreement with previous results (26). However, no inversions were detected in the same transfections of xrs-6 and sc3T3 cells which gave rise to abundant hybrid joints (data not shown). Therefore, hybrid joints are formed far more efficiently than inversions in xrs-6 and sc3T3 cells. These data indicate that neither the absence of functional Ku86 (xrs-6 cells) nor the presence of a mutant form of DNA-PK_{CS} (sc3T3 cells) significantly impairs the efficiency of hybrid joint formation.

Most hybrid joints contain intact signal and coding sequences. To survey the structure of the population of hybrid joints, PCR products were subjected to digestion with the restriction enzyme *Bam*HI, which will only digest PCR products of hybrid joints that have lost fewer than 2 nucleotides from the coding element. The majority of the products from all three cell lines were cleaved with *Bam*HI, indicating that hybrid joints rarely suffer the loss of more than 1 nucleotide from the coding end (data not shown).

To examine the structure of hybrid joints in more detail,

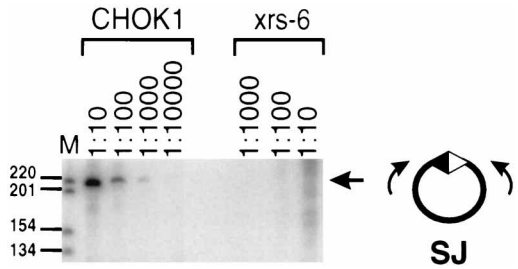


FIG. 5. Signal joints are not detected in xrs-6 cells. Signal joints (SJ) were detected by circular PCR using the DR55 and ML68 primers (curved arrows). The blot was hybridized with an end-labeled oligonucleotide probe (DR73). Note that the PCR products generated by using high levels of DNA from xrs-6 cells (1:10 dilution) are observed in the absence of RAG expression vectors (data not shown), indicating that they are not derived from signal joints. Numbers to the left of the gel are marker (M) sizes (in base pairs).

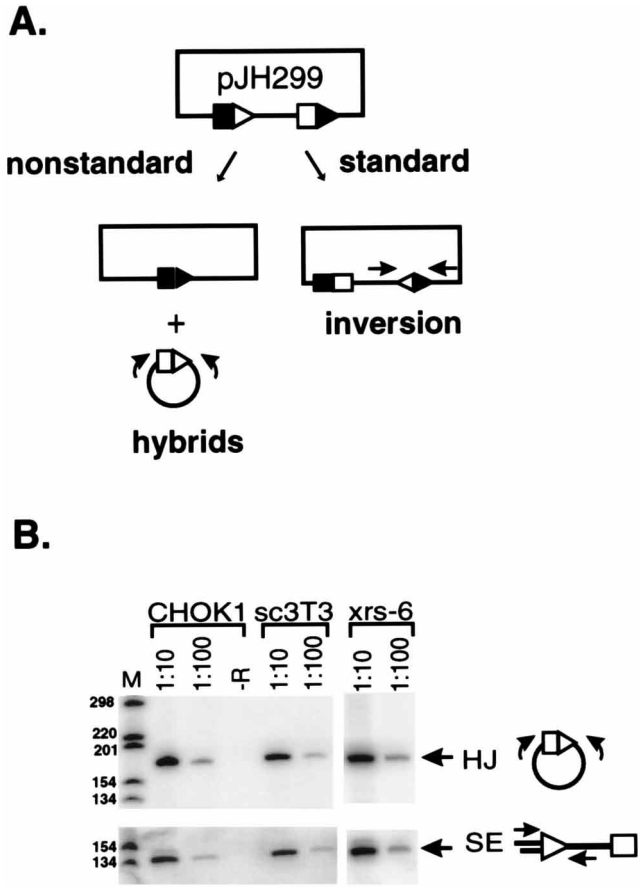
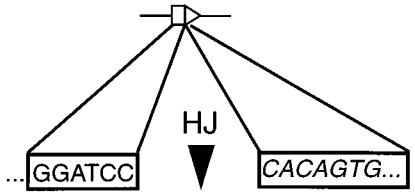


FIG. 6. Efficient formation of hybrid joints in xrs-6 cells. (A) Schematic diagram of pJH299 substrate and hybrid formation. Cleavage of pJH299 produces both signal and coding ends on the plasmid and the excised products. The joining of the ends generates standard junctions (inversions) on the plasmid and nonstandard junctions (hybrid joints) on both the plasmid and the excised product. (B) Hybrids are formed in xrs-6 cells. Hybrid joints (HJ) on the excised product were detected by circular PCR using DR55 and ML68 primers. The blot was probed with the hybrid junction-specific probe DR98, which hybridizes to 12 nucleotides of the coding end and 12 nucleotides of the signal end (SE). To detect 12-spacer signal ends on the excised product, LMPCR was performed with DR55 and DR20 PCR primers. The blot was hybridized with a radiolabeled oligonucleotide (DR69). −R indicates the absence of RAG in the transfection. 1:10 and 1:100 represent dilutions from 1/50 of the DNA recovered from each transfection performed as described above. For detection of hybrid joints, 1 μl of each dilution was used. To detect signal ends, 1/20 of each dilution was used. Numbers to the left of the gel are marker (M) sizes (in base pairs).

PCR products were cloned and analyzed by nucleotide sequencing. The results (Fig. 7) demonstrate that the majority of hybrid joints in all three cell lines form without loss of nucleotides from either the coding or signal ends. The next most common class of junctions contained single nucleotide additions, which cannot be attributed to terminal deoxynucleotidyl transferase since the fibroblasts used in this study do not express this activity. The single extra G residues present in many junctions are a specific type of junctional insert, termed P nucleotides, which are derived from hairpin opening (23, 32). Two other single-nucleotide insertions (of a T residue and a C residue) were observed (Fig. 7). Insertions of AT base pairs are commonly seen in junctions produced by end joining in fibroblasts (40) and can be explained by the addition of a nontemplated A residue by DNA polymerase (8). It is more difficult to account for the apparent addition of a single C residue in the absence of terminal deoxynucleotidyl transferase. One possibility is that this residue resulted from imprecise cleavage at a point 1 nucleotide inside the heptamer sequence, resulting in an extra C at the coding end. Such events have been described in rearrangements of TCR genes (5).

Although the majority of junctions in all three cell lines retained all nucleotides from both ends, deletions from both coding and signal ends were occasionally observed in CHOK1 and xrs-6 cells. Loss of nucleotides from the coding ends is characteristic of hybrid joints; however, deletions from signal ends have also been observed in hybrid joints from both wild-type (26) and *scid* cells (28). Although the two largest deletions in our collection were derived from xrs-6 cells, losses of similar numbers of nucleotides from signal ends have been reported in hybrid joints from wild-type lymphoid cell lines (26), suggesting that these deletions are not necessarily related to Ku deficiency. From these data, we conclude that neither the effi-



	(Bam)		RSS	n
A. CHOK1 (30)	0		0	14[1]
	0	G	0	9[2]
	-1	T	0	1
	-4		-3	1
	-2		-7*	1
	-1		0*	2
	-4		0	1
	-2		0	1
B. xrs-6 (27)	0		0	16[3]
	0	G	0	6[4]
	0	C**	-13	1
	-4		-15	1
	-4		-27	1
	-11		-2	1
	-13		0	1
C. sc3T3 (14)	0		0	8
	0	G	0	6

FIG. 7. Nucleotide sequence analysis of hybrid joints. PCR products containing hybrid joints were cloned and sequenced. The solid triangle designates the position of the junction. Letters in boldface type indicate extra nucleotides. Circled letters indicate presumptive P nucleotides. Numbers in column n are the numbers of junctions with the indicated sequences. The number of nucleotides deleted from each end is indicated in the columns beneath the corresponding end sequences. The numbers in parentheses beneath the cell line designation (CHOK1 [A], xrs-6 [B], and sc3T3 [C]) indicate the numbers of junctions sequenced. A single asterisk indicates that the end from which the deletion occurred cannot be assigned due to a 1-nucleotide homology. In these cases deletions were arbitrarily assigned to the coding end. Double asterisks indicate an extra C that could be due to imprecise cleavage between the coding flank and the heptamer of the RSS. Superscript numbers designate junction sequences that were derived from multiple independent transfections as follows: 1, three transfections; 2, two transfections; 3, four transfections; 4, three transfections.

ciency of formation of hybrid joints nor their structure is significantly affected in Ku86-deficient or *scid* fibroblasts.

DISCUSSION

Ku86 and processing of V(D)J recombination intermediates. Here we report the first analysis of V(D)J recombination intermediates in a cell line bearing a mutation that affects V(D)J recombination. Our data demonstrate that while formation of signal joints is severely impaired in xrs-6 cells, ligatable signal ends are present at levels similar to those in wild-type CHOK1 cells. These results closely parallel those obtained from Ku86-deficient mouse thymocytes, in which intact signal ends resulting from cleavage at the TCR δ locus are present at levels similar to those seen in wild-type thymocytes (58). Since the efficiency of signal joint formation is severely impaired in the absence of Ku86, one might expect a large accumulation of signal ends in the mutant cells. It is not yet clear why this is not observed in either mouse thymocytes or fibroblast cell lines. However, it is noteworthy that TCRδ coding ends, which accumulate in Ku86-deficient and *scid* thymocytes, are present at levels similar to those of signal ends (42, 58, 59). This may reflect some intrinsic limitation on the abundance of broken ends in this system.

Our results show that all detectable signal ends in xrs-6 cells are blunt and full length. These data clearly demonstrate that Ku86 is not required either for cleavage at the RSS or for protection of the resulting signal ends. Extensive nucleotide sequence analysis of hybrid joints also supports the conclusion that Ku86 is not required for end protection, as the majority of these junctions contain intact coding and signal sequences. Since Ku70 protein levels are reduced to undetectable levels in xrs-6 cells (6), it is unlikely that Ku70 is required for end protection or for hybrid joint formation.

These results, obtained in *xrcc5* mutant fibroblasts, are in agreement with our previous studies of endogenous TCR gene segments in mouse thymocytes, which found similar levels of signal ends at the TCRβ and -δ loci in wild-type and Ku86-deficient mice (58). Analysis of an independently derived *xrcc5* knockout mouse previously showed that signal ends could not be detected at the immunoglobulin heavy-chain locus in thymocytes of these animals (36). However, signal ends have recently been detected at the TCRδ locus in thymocytes from those Ku86$^{-/-}$ mice (35a). Together, these data indicate that impaired formation of signal joints despite the presence of abundant, intact signal ends appears to be a general feature of V(D)J recombination in Ku86-deficient cells. Although the reason for the lack of signal ends at the heavy-chain locus has not been established, one possibility is that this locus may not be recombinationally active in Ku-deficient thymocytes.

The absence of Ku86 could impair joining of signal ends either by rendering the joining activities defective or by impeding the access of the joining machinery to the ends. Two independent investigations have found that ligation of extrachromosomal plasmid DNA molecules is not substantially impaired in xrs-6 cells (19a, 27), indicating that the failure to join signal ends in this cell line is not simply due to a defect in general cellular end-joining activities. This conclusion is supported by our observation that formation of hybrid joints is not impaired in Ku86-deficient cells. Furthermore, our detection of full-length signal ends and abundant, undeleted hybrid joints in xrs-6 cells suggests that the signal ends are protected from degradation in the absence of Ku86. Together, these data suggest that in Ku86-deficient cells V(D)J recombination intermediates are generally inaccessible to both end-modifying activities and to the joining machinery responsible for generating standard junctions.

The identities of the factors responsible for protecting the broken ends remain unknown. However, precedents for the persistence of stable DNA-protein complexes after cleavage are found in transposition reactions such as Mu (52) and Tn*10* (14). For example, bacteriophage Mu transposition proceeds through a series of intermediates in which the recombinase (MuA protein) is tightly bound to the Mu end sequences (1, 34, 52). The final intermediate, which is remarkably stable, is disassembled by a molecular chaperone, ClpX, which specifically interacts with MuA and promotes access of the cellular replication machinery (22, 24).

A similar situation may occur in V(D)J recombination. Several lines of evidence strongly suggest that efficient cleavage requires formation of a DNA-protein complex (termed a synaptic complex) involving both RSS (9, 50, 51, 57). Perhaps this complex, which is likely to include the RAG proteins, persists after DSB formation, sequestering all four ends from both joining and degradation activities. We have suggested that Ku may be required to disassemble or remodel the complex, making the ends available for joining (58). This view, which is supported by the experiments reported here, contrasts sharply with the original end protection model which suggested that Ku might be required for protection of DNA ends.

Inefficient formation of standard junctions in the absence of Ku. Previous studies have shown that both signal and coding joints can form at low levels in xrs-6 cells (38, 55), and rare coding joints have been observed in Ku86-deficient mice (36, 58), indicating that formation of some standard junctions can, inefficiently, bypass the requirement for Ku86. While no coding joint sequences from Ku86-deficient cells or mice have been reported, signal joints isolated from xrs-6 cells generally exhibit deletions (38, 55), which are distinctly unusual in wild-type cells. The disassembly model described above suggests two simple explanations for these deletions. First, the requirement for Ku could be bypassed by complexes that fall apart nonspecifically. Since the signal ends produced by this pathway would not be protected by the complex, they should be treated by the joining machinery as free ends. Deletions at junctions derived from blunt ends introduced directly into fibroblast nuclei by microinjection are observed far more frequently than deletions at signal joints (44). These deletions presumably reflect the actions of end-processing activities. Similar processing of free signal ends could account for the short deletions frequently seen in signal joints from Ku-deficient cells. Random endonucleolytic cleavage would provide a second mechanism to allow free ends to escape from the complex. Such events could generate much larger deletions, such as those observed at the antigen receptor loci in *scid* mice (30, 49).

Hybrid joints bypass the requirement for Ku and DNA-PK. Hybrid joints are thought to result from mistakes in the handling of broken ends, resulting in joining a coding end to a signal end (26). This hypothesis is supported by the presence of characteristic junctional modifications such as loss and/or addition of nucleotides at some hybrid joints (26, 28). Our data are in agreement with this model, as a number of the hybrid joints reported here exhibit both loss and addition of nucleotides, including presumptive P nucleotides, which are thought to be derived from the opening of hairpins (32, 42). Thus, hybrid joints are apparently formed from the same intermediates (hairpin coding ends and blunt signal ends) as are standard products. However, our results show that in xrs-6 cells, where formation of standard products is severely defective, these ends can still be joined efficiently to form hybrid joints. We obtained similar results in *xrcc7* mutant fibroblasts, in

agreement with previous work with *xrcc7* mutant lymphoid cells, which are severely defective for joining coding ends (28). Furthermore, our data indicate that joining in both mutant cell lines generally proceeds without excessive loss of nucleotides from the ends, in sharp contrast to the extensive deletions observed at standard junctions formed in these cells. Hybrid joints that retain all nucleotides of both ends have also been observed in endogenous antigen receptor rearrangements in Ku-deficient mice (our unpublished data).

The notable failure of *xrcc5* and *xrcc7* mutations to substantially affect the abundance or the structure of hybrid joints indicates that the pathways responsible for formation of these nonstandard products bypass the normal requirement for Ku and DNA-PK_{CS}. We have considered two classes of models to explain this phenomenon. One possibility is that hybrid joints are normally derived from improperly assembled or prematurely disrupted synaptic complexes, which might not require specific disassembly to promote access of the joining machinery. Nonspecific disruption of the complexes could allow nucleases to act on signal ends, which would normally be protected. This is consistent with the loss of nucleotides from signal ends observed in some hybrid joints from fibroblasts (this work) as well as from lymphoid cell lines (26, 28).

Another possibility is that formation of hybrid joints might not require disassembly of the synaptic complex. Perhaps RAG proteins present in the complex mediate joining, using the same chemical steps normally employed for RSS cleavage. In the final step of the RSS cleavage reaction, the RAG proteins use the 3′ hydroxyl group formed by nicking at the coding-RSS border to attack the phosphodiester bond on the opposite strand, forming a blunt signal end and a hairpin coding end (Fig. 8A). We suggest that RAG proteins bound to signal ends in the synaptic complex may simply reverse this reaction, using the 3′ OH of the signal end to attack a phosphodiester bond of a hairpin coding end (Fig. 8B and C). This reaction could either regenerate the original substrate or, if a different coding end were used, generate a hybrid joint. This proposed RAG-mediated joining reaction is strictly analogous to disintegration reactions carried out by retroviral integrases, which involve reversal of the initial DNA cleavage-ligation steps (7). The same chemical mechanism is utilized by the RAG nuclease and the retroviral integrases, and both systems perform similar reactions, including direct transesterification with inversion of chirality, alcoholysis, and hairpin formation (discussed in reference 56). Thus, it seems reasonable to suppose that the RAG nuclease might also perform a reaction analogous to disintegration.

In its simplest form, the proposed RAG-mediated joining mechanism proceeds conservatively, without loss or addition of sequence information. Thus, hybrid joints formed by this pathway should not suffer loss of nucleotides from either the signal or coding ends. Our data are consistent with this prediction, as 59 of the 71 junctions (83%) exhibited no loss of nucleotides. Furthermore, the model can simply account for the presence of specific extra nucleotides (P nucleotides) at hybrid joints. Attack of the 3′ OH precisely at the "tip" of the hairpin (between the two terminal nucleotides) would generate a perfect hybrid joint (Fig. 8B). However, attack on the bond immediately 5′ or 3′ to the terminal position would generate a hybrid joint containing a 1-nucleotide inverted repeat (Fig. 8C), as observed in 30% of the junctions in our collection.

RAG-mediated joining, which might be promoted by "stuck" synaptic complexes present in the absence of Ku86, could account for the majority of hybrid joints in Ku86-deficient cells. The less frequent hybrid joints with deletions could be accounted for by a pathway that involves free ends, similar to the bypass pathway that allows formation of rare signal joints with deletions in Ku-deficient cells. Further experiments will be required to more fully explore the pathways responsible for formation of hybrid joints. Understanding the surprising ability of these mechanisms to bypass the normal requirements for Ku and DNA-PK should provide important insights into the joining mechanisms used in V(D)J recombination as well as into the roles of Ku and DNA-PK in formation of standard junctions.

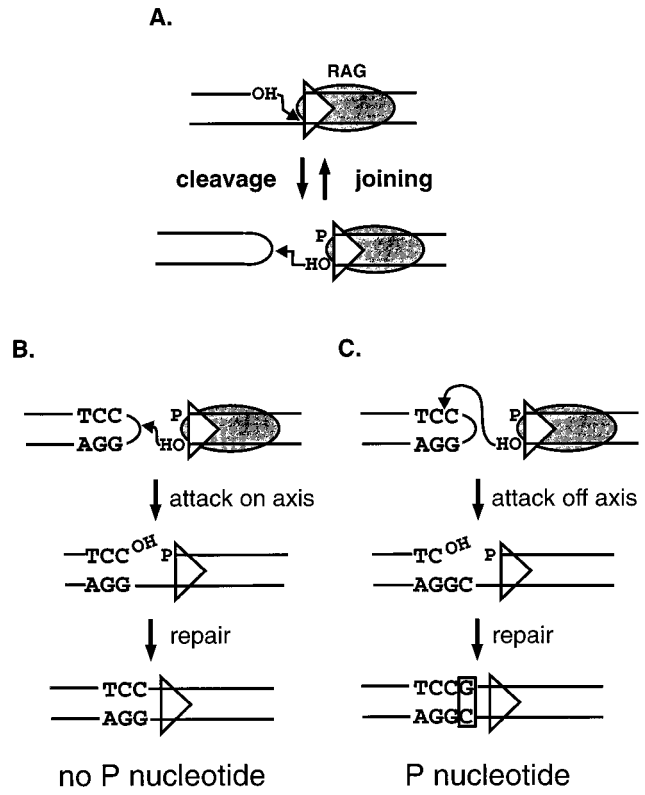


FIG. 8. A RAG-mediated joining model. (A) RAG-mediated cleavage and joining reactions are illustrated. In the first step of cleavage (not shown), the RAG nuclease (shaded oval) introduces a nick precisely between the RSS (triangle) and the coding flank (31). This produces a 3′ OH which is used in the second step to attack the phosphodiester bond on the bottom strand, forming a hairpin coding end and a blunt signal end (31). We propose that this reaction can run in reverse, using the 3′ OH of the signal end as a nucleophile to attack a phosphodiester bond of the hairpin coding end, producing a phosphodiester bond linking the coding and signal ends. This joining reaction could rejoin the signal end either to the original coding end, regenerating the substrate, or to the other coding end, producing a hybrid junction. (B) Attack of the 3′ OH on the axis of symmetry of the hairpin (between the two terminal nucleotides) would produce a junction that contains no extra nucleotides. Presumably the nicked product generated by the joining reaction could subsequently undergo ligation (repair). (C) Attack of the 3′ OH at positions away from the axis of symmetry would produce junctions containing extra nucleotides (P nucleotides). Attack on the bond immediately 5′ of the hairpin terminus is illustrated, although the same junction could be produced by attacking 1 nucleotide 3′ of the terminus. The initial joining reaction would produce a molecule with a gap which could be repaired subsequently.

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