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Double-strand break repair in Ku86- and XRCC4-deficient cells

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ABSTRACT

The Ku86 and XRCC4 proteins perform critical but poorly understood functions in the repair of DNA double-strand breaks. Both Ku 86- and XRCC4-deficient cells exhibit profound radiosensitivity and severe defects in V(D)J recombination, including excessive deletions at recombinant junctions. Previous workers have suggested that these phenomena may reflect defects in joining of the broken DNA ends or in protection of the ends from nucleases. However, end joining in XRCC4-deficient cells has not been examined. Here we show that joining of both matched and mismatched DNA ends occurs efficiently in XRCC4-deficient cells. Furthermore, analysis of junctions shows that XRCC4 is not required to protect the ends from degradation. However, nucleotide sequence analysis of junctions derived from joining of mismatched DNA ends in XRCC4-deficient cells revealed a strong preference for a junction containing a 7 nt homology. Similar results were obtained in Ku86-deficient cells. These data suggest that in the absence of XRCC4 or Ku86, joining is assisted by base pairing interactions, supporting the hypothesis that these proteins may participate in aligning or stabilizing intermediates in end joining.

INTRODUCTION

The repair of double-strand DNA breaks (DSB) generated by ionizing radiation, chemical agents or other means is critical for cell survival. Mammalian cells are proficient at joining DNA ends and both simple ligation (joining of blunt ends or ends with complementary single-stranded extensions) and joining of mismatched termini occur efficiently (1–4). Joining appears to involve at least two distinct mechanisms: one that does not depend upon base pairing interactions (for example blunt end ligation) and a second pathway that employs short sequence homologies to direct joining (3,4). These mechanisms are not unique to mammals, as they have been observed in *Xenopus laevis* (5–8) and in *Saccharomyces cerevisiae* (9,10).

Studies in *Xenopus* egg extracts have shown that joining of mismatched ends involves a process in which the two termini are held together by non-covalent interactions (including pairing of short sequence homologies) while a 3′-OH from one terminus is used as a primer to direct repair synthesis, followed by ligation (5–7). Based on these data, it has been suggested that an alignment factor may function to appose the two ends (7). Similar joining events, both with and without short sequence homologies, have been observed using extracts from mammalian cells (11,12). However, the precise mechanism(s) used for joining and the identities of the proteins involved remain unknown.

Analysis of radiosensitive mutant hamster cell lines with defects in DSB repair led to the identification of several factors that may be involved in end joining (13,14). One particularly attractive candidate is Ku, an abundant, heterodimeric DNA end-binding complex composed of 70 and 86 kDa subunits (termed Ku70 and Ku86), which binds to a variety of end configurations, including blunt ends and termini with 5′ or 3′ single-stranded extensions (15–18). The catalytic subunit of DNA-PK (DNA-PKcs) is activated when directed to DNA lesions, including ends, by Ku. Once activated, DNA-PKcs can phosphorylate a variety of protein substrates which may serve to signal the presence of DNA damage (15,19–21).

The suggestion that Ku may play a role in DNA end joining is supported by analysis of hamster cell lines bearing mutations in the *XRCC5* gene, which encodes the 86 kDa Ku subunit. Cells bearing *xrcc5* mutations, such as the xrs-6 cell line, are devoid of Ku end-binding activity and do not express detectable levels of the Ku70 subunit, whose stability requires the presence of Ku86 (22,23). These mutants are hypersensitive to ionizing radiation or other agents that cause DSB (24) and are severely defective for the joining of broken DNA ends created during V(D)J recombination (13,14,25,26). Furthermore, a particular class of V(D)J junctions, termed signal joints, isolated from xrs-6 cells frequently contain abnormal deletions (13,14). Based on these observations, along with the knowledge that the Ku heterodimer binds to DNA ends, we and others suggested that a major role of Ku might be to protect DNA ends from degradation (19,21,25,27–31). This interpretation receives some support from a study of plasmid end joining in Ku86-deficient cells, however, the effects were variable, depending on the time of DNA harvest after transfection

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(31). Our recent analysis of V(D)J recombination intermediates indicates that those broken ends are intact in Ku86-deficient cells, arguing against a role for Ku in end protection (26,32).

Analysis of another mutant hamster cell line (XR-1) with defects in DSB repair and V(D)J recombination led to the isolation of the *XRCC4* gene (33). The predicted sequence of the XRCC4 protein provided no information about its function (33). However, both DNA-PK activity (19) and DNA end-binding by Ku (27,28) are normal in XR-1 cells, indicating that the XRCC4 protein is not an essential component of the DNA-PK holoenzyme. DNA end joining has not been studied in XRCC4-deficient cells. However, analysis of V(D)J recombination products isolated from XRCC4-deficient cells has led to the suggestion that XRCC4 may protect DNA ends from degradation or help align DNA termini during joining (14,33). Another role in joining is suggested by recent data indicating that XRCC4 interacts with DNA ligase IV (34,35). Although the functional significance of this interaction remains to be established, it raises the intriguing possibility that at least one function of XRCC4 might be to recruit or stimulate ligase activity.

Analysis of V(D)J recombination products and intermediates has provided some tantalizing clues about the functions of Ku and XRCC4 in the metabolism of broken DNA ends. However, it is important to note that the mechanisms responsible for joining V(D)J recombination intermediates remain unknown. Furthermore, recent evidence indicates that the V(D)J recombinase complex may participate in both protection (36) and joining (37) of V(D)J recombination intermediates. Therefore, it is critical to study the effects of these mutations on end joining in the absence of V(D)J recombination.

In this study we examined the ability of Ku86- and XRCC4-deficient mutant hamster cell lines to join DNA ends using a plasmid rejoining assay that has been employed previously to study end joining in mammalian cells and yeast (3,38,39). Our results indicate that Ku86 and XRCC4 are not required to protect DNA ends from degradation. Furthermore, nucleotide sequence analysis of junctions from XRCC4-deficient cells revealed a striking predominance of short sequence homologies. Conversely, overexpression of human XRCC4 in the mutant cells caused a substantial reduction in the proportion of junctions with short sequence homologies compared with wild-type control cells. These data suggest that the XRCC4 protein plays a role in directing end joining and that alternative joining processes requiring base pairing are used in its absence.

MATERIALS AND METHODS

Cell lines and cell culture

The XR-1 cell line is XRCC4-deficient owing to a complete deletion of the *XRCC4* gene (33). The xrs-6 cell line fails to express functional Ku86 protein because of a splice site mutation which results in a 13 nt insertion and a frameshift (40). Control cell lines included the parental wild-type cell line CHOK1 4364 (41) (hereafter termed CHOK1), another wild-type CHO cell line termed RMP41 (42) and the XR-1-TR cell line. XR-1-TR was derived from XR-1 by stable transfection of a human wild-type *XRCC4* cDNA, restoring V(D)J recombination and resistance to γ-irradiation to normal. The XR-1-TR transfectant was obtained by subcloning the *XRCC4* cDNA (33) into pcDNA 3.1 (InVitrogen), transfecting this construct into XR-1 cells and selecting for G418-resistant clones which were also resistant to γ-irradiation. Cell lines 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. For XR-1-TR cells, Geneticin (Gibco BRL) (final concentration 400 μg/ml) was added to the medium. V(D)J recombination assays using transiently transfected substrates (26,43) were performed to verify that all cell lines expressed the appropriate wild-type or mutant phenotype (data not shown).

DNA substrates

Plasmid pJH452, which lacks V(D)J recombination signal sequences but is otherwise identical to pJH299 (44), was linearized by digestion with the indicated restriction endonucleases. To assay for simple ligation, the substrate was linearized with either *Bam*HI or *Sal*I (both of which cut only once in the plasmid) or with *Cla*I, which cuts twice. To study joining of non-complementary termini, double digestion with *Bam*HI and *Sal*I was performed. After digestion, DNA was purified by phenol extraction and ethanol precipitation. All linearized substrates were analyzed by agarose gel electrophoresis and by transformation into *Escherichia coli*, which demonstrated that the completeness of digestion was >99%.

Assay for end joining

Cells were transiently transfected with 4–7 μg of linear plasmids; simultaneous, parallel transfections were performed using the same quantities of supercoiled plasmids. Transfections were done by the calcium phosphate method and DNA was harvested after 38–48 h as previously described (43). Since a replicating plasmid was used, transfection efficiency was readily verified by digesting harvested DNA with *Dpn*I, which cleaves bacterially methylated DNA that has not replicated in mammalian cells, followed by bacterial transformation as previously described (45).

End joining was measured by transforming DNA recovered from transfections into competent *E.coli* DH5α cells. Transformants arising from rejoined circular plasmids were selected on LB agar plates containing ampicillin (100 μg/ml). End joining efficiency was determined as described previously (3,38) by dividing the number of transformants arising from (circularized) linear plasmids by the number of transformants arising from parallel transfections of undigested plasmid DNA, after normalizing for transfection efficiency. All transfections were performed a minimum of three times, with similar results.

Analysis of junctions

Ampicillin-resistant colonies resulting from end joining were screened for resistance to chloramphenicol by replica plating onto medium containing chloramphenicol (40 μg/ml). Plasmid DNA was sequenced using a Sequenase v.2.0 sequencing kit (US Biochemical, Cleveland, OH). Alternatively, PCR products obtained from chloramphenicol-resistant colonies were sequenced using a Thermosequenase sequencing kit (Amersham, Arlington Heights, IL). DR1 was used as sequencing primer. For amplification of end-joined junctions, PCR primers were chosen that can amplify any junctions that lie within the minimal region required for chloramphenicol resistance. Thus, primer DR257 is situated upstream of the p*lac* promoter and primer DR100 (26) is located within the essential region of the *cat* gene.

To determine the fidelity of joining of complementary termini, ampicillin-resistant colonies were used for either plasmid isolation or PCR amplification and the plasmids or PCR products were tested by digestion with the relevant restriction enzyme (*Bam*HI or *Sal*I). PCR primers used for amplification of these products were DR99 (26) and DR100.

Oligonucleotides

DR1, 5′-CAACGGTGGTATATCCAGTG-3′;
DR257, 5′-CAATACGCAAACCGCCTCTCC-3′.

RESULTS

Faithful, efficient end joining in Ku86- and XRCC4-deficient cells

The substrate plasmid we used (Fig. 1) is capable of replicating in both mammalian cells and in *E.coli* and contains two bacterial drug resistance markers. Substrates were linearized by digestion with restriction enzymes which cleave at sites located in a region not essential for plasmid maintenance or replication, allowing a variety of joining events to be recovered. This strategy is similar to that used in other studies of end joining in mammalian cells (2,4,46,47) and yeast (38,39). Substrates digested with either *Sal*I or *Bam*HI were used to study joining of complementary termini. As shown in Table 1, the proportion of ampicillin-resistant colonies arising from transfections using the linearized substrate relative to the circular control plasmid was similar in the mutant and control cell lines. Thus, the efficiency of complementary end ligation is not substantially impaired in XRCC4- or Ku86-deficient cells. To examine joining of mismatched ends, the substrate plasmid was digested with both *Sal*I and *Bam*HI, producing termini with non-complementary 5′ extensions. The proportion of ampicillin-resistant colonies arising from this substrate was not decreased in the mutant cell lines relative to the control cell lines (Table 1). These data indicate that the efficiency of end joining is not significantly impaired in cells lacking either Ku86 or XRCC4.

The experiments described above show that Ku86 and XRCC4 are not required for joining either complementary or non-complementary DNA termini. However, the data do not address the possibility that the structure of the junctions formed in the absence of these proteins might be altered. For example, if XRCC4 or Ku86 were required to protect broken DNA ends, one would expect a higher frequency of deletions at junctions formed in the mutant cells. To examine this possibility, we first measured the fidelity of joining by determining the frequency with which ligation of complementary ends regenerated the original restriction site. Individual colonies derived from *Sal*I- or *Bam*HI-digested substrates were isolated and the plasmids were tested for the presence of the *Sal*I or *Bam*HI site, respectively. As shown in Table 2, no consistent decrease in the proportion of junctions that regenerate the original restriction site was observed in the mutant cell lines. In fact, a 2- to 3-fold *increase* in the percentage of junctions containing the restriction sites was observed in the XR-1 cell line. These data indicate that precise rejoining of DNA ends is not impaired in the mutant cells, indicating that neither XRCC4 nor Ku86 are required for efficient, accurate ligation of complementary DNA termini.

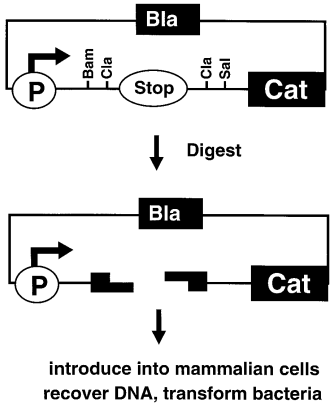

Figure 1. Assay for DNA end joining. The substrate plasmid used for end joining assays is diagrammed. The plasmid can replicate in both bacteria and mammalian cells and contains a β-lactamase gene (Bla) and a chloramphenicol acetyltransferase gene (Cat) separated from its promoter (P) by a prokaryotic transcription terminator sequence (stop). Substrates for end joining were created by digestion at the *Bam*HI, *Cla*I and *Sal*I restriction sites.

Table 1. Efficiency of end joining

Cell line	Genotype	Reference	Complementary[a]	Non-complementary[b]
CHOK1 4364	Wild-type	41	11.7 ± 8.1 (4)	3.1 ± 2.4 (4)
RMP41	Wild-type	42	16.5 ± 1.8 (3)	20.0 ± 2.5 (3)
xrs-6	*xrcc5*	24	7.8 ± 2.4 (4)	20.0 ± 8.7 (6)
XR-1	*xrcc4*	58	12.9 ± 8.6 (4)	8.7 ± 5.5 (8)
XR-1-TR	+ hXRCC4 cDNA	This work	nd[c]	9.1 ± 4.5 (4)

[a]The average of the end joining efficiency was measured using substrates singly digested with either *Bam*HI or *Sal*I, since these two enzymes gave similar results. End joining efficiencies were calculated as described in Materials and Methods and are shown ±1 SD. Numbers in parentheses denote the number of independent transfections analyzed.
[b]End joining efficiency observed using substrates doubly digested with *Bam*HI and *Sal*I.
[c]Not determined.

Table 2. Fidelity of joining complementary termini

	Perfect junctions[a]	
	*Bam*HI	*Sal*I
CHOK1	42% (15/36)	36% (13/36)
xrs-6	22% (17/77)	34% (27/80)
XR-1	57% (32/56)	61%(34/56)
XR-1-TR	25% (17/68)	nd

[a]As determined by sensitivity to the restriction enzyme used to linearize the substrate plasmid. Numbers in parentheses indicate number of sensitive colonies/total number of colonies screened.

Lack of excessive deletions at junctions isolated from Ku86- and XRCC4-deficient cells

Since the joining of complementary ends is a simple ligation event which in principle requires only a DNA ligase activity, we examined joining of non-complementary termini, a more complex reaction that requires the action of several activities, perhaps including alignment factors (7). To search for junctions containing deletions we employed a simple screen for circularized plasmids which have lost >50–80 nt from either end. As illustrated in Figure 1, the substrate contains a chloramphenicol acetyltransferase (*cat*) gene separated from its promoter by a transcription terminator so that the intact plasmid does not confer resistance to chloramphenicol. Double digestion with *Bam*HI and *Sal*I results in loss of the intervening DNA fragment containing the terminator. Thus, if rejoining proceeds with minimal loss of nucleotides from either end, the circularized plasmid confers chloramphenicol resistance. However, loss of >50 nt from the *Bam*HI end or 80 nt from the *Sal*I end would result in loss of drug resistance due to deletion of essential nucleotides from the coding sequence of the *cat* gene or the promoter, respectively. Plasmids containing such deletions were identified by replica plating the bacterial transformants onto medium containing ampicillin or ampicillin plus chloramphenicol.

Table 3. Frequency of deletions formed by joining of non-complementary termini

	Cam^R colonies (%) (*n*)[a]
CHOK1	38 ± 11 (4)
xrs-6	32 ± 8.4 (5)
XR-1	37 ± 6.6 (5)
XR-1-TR	29.2 ± 8.6 (4)

[a]Joining events that delete <~50 nt from *Bam*HI end and <~80 nt from the *Sal*I end will preserve both the coding sequence of the *cat* gene and the p*lac* promoter, respectively, resulting in chloramphenicol-resistant (Cam^R) colonies.

As shown in Table 3, rejoined plasmids recovered from both mutant and wild-type cell lines gave similar proportions of ampicillin-resistant, chloramphenicol-sensitive colonies, indicating that the frequency of deletions measured by this assay is not increased in Ku86- or XRCC4-deficient cells. Deletions of more than several hundred nucleotides would result in loss of the ability to confer ampicillin resistance by crippling other essential regions of the plasmid. However, the frequency of ampicillin-resistant products derived from joining of the doubly digested substrate plasmid was similar in the mutant and wild-type cell lines (Table 1). Together, these results indicate that the frequency with which substantial numbers of nucleotides are lost from the ends is not increased in cells lacking Ku86 or XRCC4 proteins, arguing against a requirement for these proteins in protecting broken DNA ends from exonucleolytic degradation.

End joining is frequently directed by a 7 nt homology in Ku86- and XRCC4-deficient cells

To examine the effects of Ku86- and XRCC4 deficiency on the joining reaction in more detail, we determined the nucleotide sequences of junctions derived from joining of doubly digested substrates. Overall, 134 sequences were obtained from plasmids which conferred resistance to chloramphenicol: 38 from XR-1, 34 from xrs-6, 21 from the XRCC4-complemented (XR-1-TR) cell line and 41 junctions from the wild-type control cell lines. Junctions from wild-type and XR-1-TR cells (Fig. 2) contained deletions ranging from 0 to 77 nt. In junctions from xrs-6 cells (Fig. 3), deletions ranged from 0 to 87 nt and in junctions from XR-1 cells (Fig. 3), deletions ranged from 6 to 102 nt.

The distribution of deletion sizes is summarized in Figure 4. While the distributions are similar in the wild-type and XR-1-TR cells, in XR-1 cells there is a favored junction (overall deletion of 33 nt) that accounts for 58% (22 of 38) of the joints. This junction was also the most common joint derived from xrs-6 cells (26%, 9 of 34 junctions). Examination of the sequence of the preferred junction revealed a 7 nt homology (Fig. 3), suggesting that the homology may have directed the joining process.

A summary of the use of junctional homologies is shown in Figure 5. Most junctions from wild-type cells (84%) exhibit homologies of 0–2 nt and only 10% of the junctions contain the 7 nt homology. In contrast, most junctions from XR-1 cells (58%) exhibit the 7 nt homology, with only 24% showing 0–2 nt homology. Note that a few junctions (8%) from XR-1 cells have 0 nt homology, indicating that joining can occur, albeit rarely, in the complete absence of sequence homology in this cell line.

The frequency of junctions from xrs-6 cells containing 0 nt homology (24%) was similar to that observed in wild-type cells. However, in contrast to the situation in wild-type cells, the predominant junction (26%) in xrs-6 cells exhibited a 7 nt homology. No junctions from the XR-1-TR cell line exhibited the 7 nt homology; notably, most junctions (76%) showed 0 nt homology (compared with 8% in the parental XR-1 cell line and 30% in wild-type cells). The increased frequency of junctions without homology observed in XR-1-TR cells compared with wild-type cells could be related to overexpression of the XRCC4 protein, as in this cell line the human *XRCC4* cDNA is driven by a strong viral promoter.

The observations described above suggest that the 7 nt homology may be used preferentially to direct joining in the mutant cells. An alternative possibility is that joining occurs randomly but with more loss of nucleotides from the ends than in wild-type cells, fortuitously producing a set of junctions with deletion end points that happen to fall within the 7 nt homology. To distinguish between these possibilities we digested the substrate plasmid with *Cla*I, a restriction enzyme with recognition sites located near the *Bam*HI and *Sal*I sites (Fig. 1). Digestion with *Cla*I produces a linear DNA molecule with 9 nt terminal

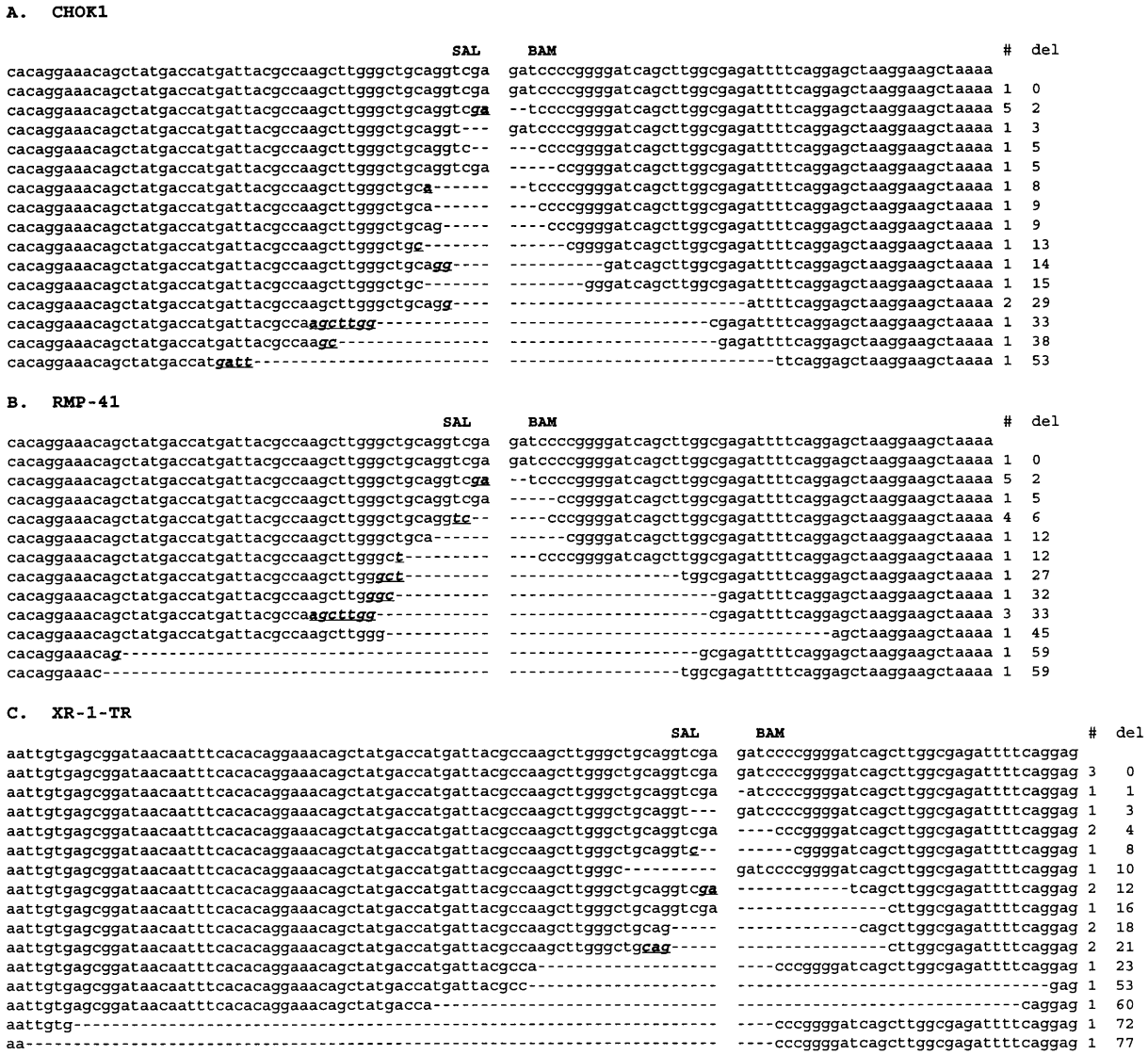

Figure 2. Nucleotide sequences of junctions created by joining *Sal*I and *Bam*HI ends in control cell lines. The sequence which would be derived from joining without loss of nucleotides from either end (i.e. derived by filling in both 5′ extensions) is shown at the top of each set of sequences. The number of times each sequence was represented in the data set is indicated in the column marked #. The number of nucleotides lost from both ends combined is shown in the column marked del. Dashes indicate nucleotides lost. Sequence homologies at the junction are shown in bold and are underlined. Each data set contains sequences from at least three independent transfections.

extensions relative to the original *Bam*HI and *Sal*I ends, positioning the 7 nt homology further from the termini. Thus, if joining is directed by the 7 nt homology, the predominant junction should contain a 51 nt deletion. The efficiency of joining of the *Cla*I ends was similar in the XR-1 and control cell lines (data not shown). Sequences of junctions derived from joining of the *Cla*I substrate transfected into XR-1 are shown in Figure 6; sequences of junctions from CHOK1 and XR-1-TR cells are shown in Figure 7. As shown in Figure 8A, the most frequent junction (seven of 26 sequences, 27%) derived from this substrate in XR-1 cells contains a 51 nt deletion corresponding to the position of the 7 nt homology. It is important to point out that in this situation, while the 7 nt homology is the predominant single junction, most joining events (73%) do not use this homology. This differs from the results obtained with the *Sal*I–*Bam*HI substrate, where almost 60% of the junctions used the 7 nt homology, indicating that the position of the homology relative to the ends may influence its availability to the joining machinery. As expected, this ‘hotspot’ is not observed in CHOK1 cells (Fig. 8B). This result indicates that loss of nucleotides is not random and suggests that joining is directed by the 7 nt homology.

DISCUSSION

Neither XRCC4 nor Ku86 is required for protection or joining of DNA ends

The data presented here provide the first evidence that the XRCC4 protein is not required for accurate, efficient ligation of complementary termini or for efficient joining of mismatched

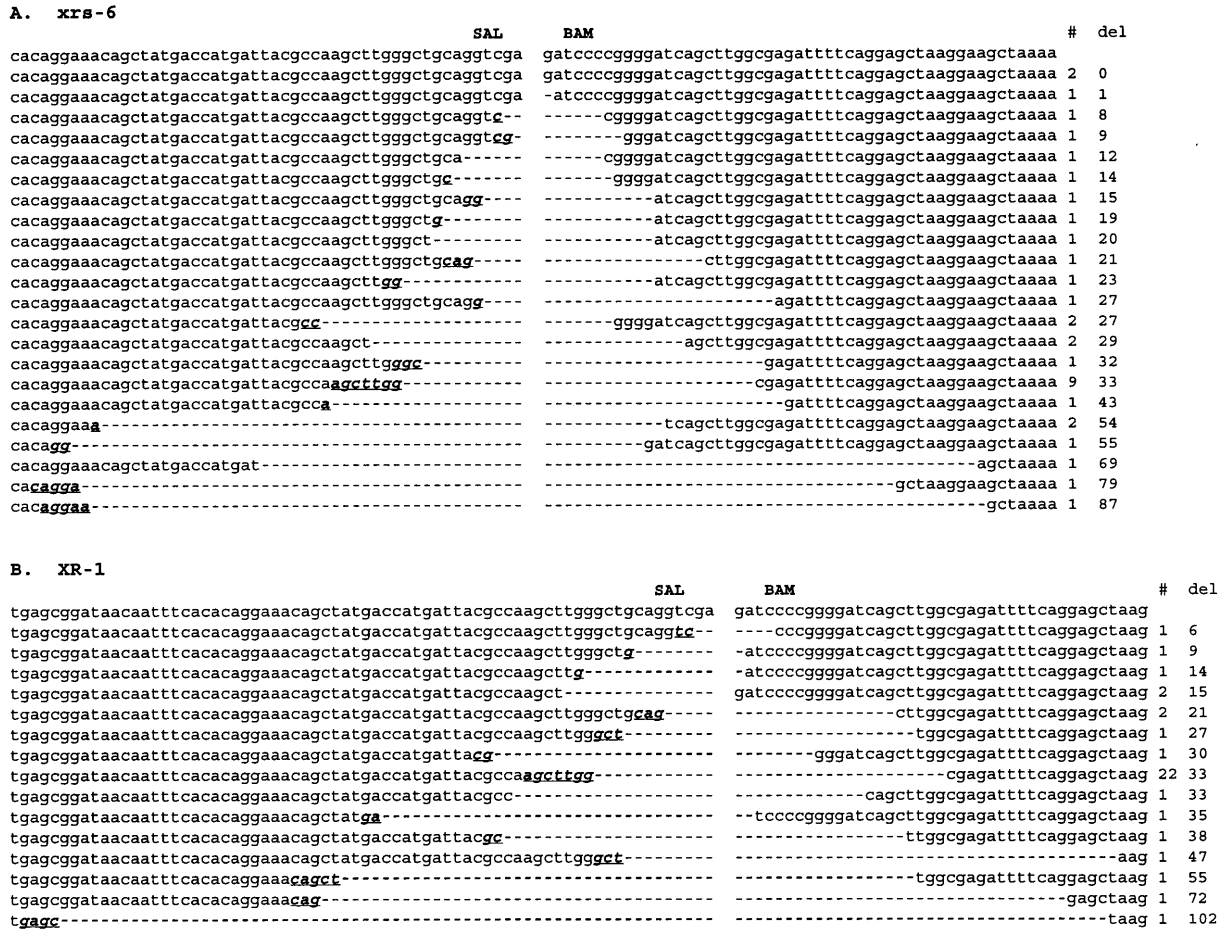

Figure 3. Nucleotide sequences of junctions created by joining *Sal*I and *Bam*HI ends in mutant cell lines. Symbols are as in Figure 2. Each data set contains sequences from at least three independent transfections. The junction containing the 7 nt homology was observed multiple times in each transfection of both xrs-6 and XR-1 cells.

ends. We also show that these reactions are not dependent upon the presence of Ku86. Since efficient activation of DNA-PK requires Ku86 (15,29), our results indicate that DNA-PK activity is not required for efficient end joining. These data are in agreement with previous studies of end joining in cell lines from scid mice (46), which express a truncated form of DNA-PKcs that may retain the ability to interact with Ku and could possess residual kinase activity (48,49). Therefore, our results indicate that the severe defects in repair of chromosomal breaks and V(D)J recombination observed in *scid*, Ku86-deficient and XRCC4-deficient cells are not caused by effects on the basic operations of DNA end joining

One hypothesis proposed to explain the hypersensitivity to DNA damaging agents and the inability to join DNA ends created during V(D)J recombination in the mutant cells is that the absence of functional XRCC4 or Ku86 proteins results in a lack of end protection, causing the broken DNA molecules to be susceptible to exonucleolytic degradation (19,21,25,27–31,33). Our results provide four lines of evidence that are inconsistent with this view. First, we observed no decrease in the recovery of ampicillin-resistant plasmids from Ku86- or XRCC4-deficient mutants, indicating that degradation of linear plasmids is not significantly increased in these cells. Second, we did not observe an increased frequency of deletions (as measured by the chloramphenicol resistance assay) resulting from joining of non-complementary termini in the mutant cell lines. Third, analysis of the fidelity of complementary end joining did not reveal an increased tendency for loss of even a single nucleotide from either end in Ku86- or XRCC4-deficient cells. Finally, nucleotide sequence analysis of numerous junctions formed by joining of non-complementary ends failed to show a significant increase in random loss of nucleotides from the ends in the mutant cell lines.

Our results from the xrs-6 cell line agree with some data from a previous study that found no major defect in plasmid rejoining at early times after introduction of a linearized, non-replicating substrate into this cell line (31). However, in that study a substantial decrease (20-fold) in the recovery of circularized plasmids was seen at late times after transfection, leading to the conclusion that end protection was defective in the absence of Ku86. Importantly, a similar decrease in the recovery of the control supercoiled substrates was also observed (31), suggesting that decreased recovery of rejoined products derived from the linear substrate may not simply result from lack of end protection, but could also reflect effects on the stability of the non-replicating plasmids. Another study (47) reported a modest (10-fold) decrease in end joining in xrs-6 cells. In agreement with our data, they observed no decrease in fidelity of joining in these cells.

Our end joining data also agree with analysis of V(D)J recombination intermediates in Ku86- and XRCC4-deficient

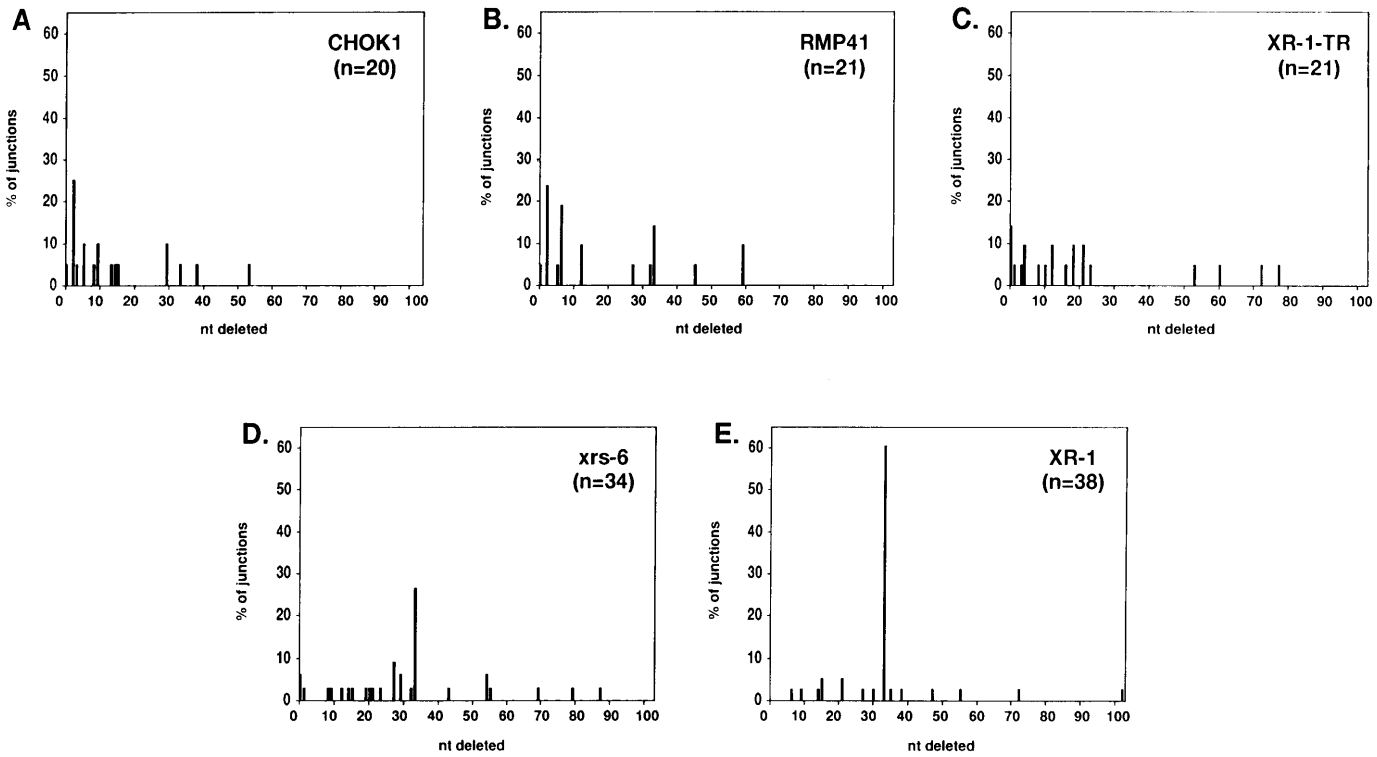

Figure 4. Distribution of deletions at junctions derived from *Sal*I and *Bam*HI ends. Each histogram shows the distribution of deletion sizes observed in the indicated cell line. The number of junctions analyzed (n) is shown. Data are from Figures 2 and 3.

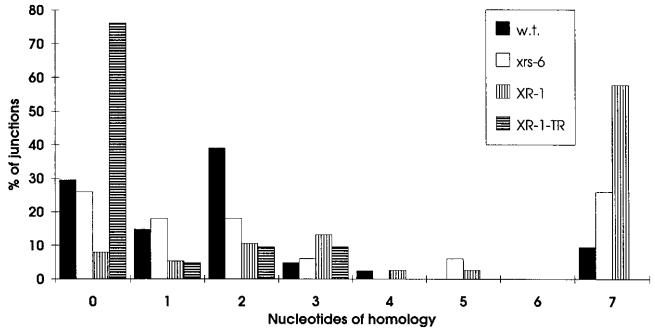

Figure 5. Short sequence homologies at junctions derived from mismatched end joining. The number of nucleotides of homology at junctions from the various cell lines are plotted. To simplify the figure, the average of data from the wild-type cell lines, CHOK1 and RMP41, is plotted as w.t. Data are from Figures 2 and 3.

cells. Broken DNA ends created during V(D)J recombination are abundant and full-length in XRCC5-deficient mice (32) and in xrs-6 cells (26), indicating that the protection of these ends does not depend upon Ku86. Furthermore, the rare coding joints isolated from XRCC5-deficient mice did not show excessive loss of nucleotides compared with coding joints from wild-type controls, again indicating that Ku86 is not required to preserve the integrity of the coding ends (50). Therefore, we conclude that Ku86 is not required to protect DNA ends created either artificially (by restriction enzyme cleavage) or during V(D)J recombination. Since the XRCC4 protein is not required to protect ends created by restriction enzyme cleavage (this work) or V(D)J recombination (51), we suggest that XRCC4 may have a more direct role in V(D)J recombination than simply to protect DNA termini from degradation (see below).

Roles of XRCC4 and Ku86 in end joining

The striking predominance of junctions containing short sequence homologies in XRCC4-deficient cells suggests that end joining is much more dependent upon base pairing interactions in the absence of XRCC4. One simple possibility is that efficient joining of mismatched ends requires formation of a complex involving the two termini and some alignment factor(s) that facilitates the joining process. The additional stability provided by formation of base paired intermediates may compensate for the lack of the alignment function, resulting in an increased proportion of junctions with short sequence homologies. Our data suggest that the 7 nt homology is a 'hotspot' for joining in the absence of XRCC4 when it is located near the ends (the 31 nt deletion accounted for nearly 60% of the junctions). However, moving this homology 20 nt further from the ends decreased its utilization substantially, as only 27% of the junctions from the *Cla*I substrate used this homology.

The hypothesis that the XRCC4 protein promotes end joining in the absence of base pairing interactions is supported by our analysis of junctions from XR-1-TR cells, which express a human *XRCC4* cDNA under the control of a strong viral promoter. In this cell line, junctions lacking homology were observed more than twice

Figure 6. Nucleotide sequences of junctions created by joining *Cla*I ends in the XR-1 cell line. Data are represented as in Figure 2. Sequences were obtained from at least three independent transfections. Junctions that regenerated the *Cla*I site were excluded from this analysis.

Figure 7. Nucleotide sequences of junctions created by joining *Cla*I ends in the CHOK1 and XR-1-TR control cell lines. Data are represented as in Figure 2. Sequences were obtained from at least three independent transfections. Junctions that regenerated the *Cla*I site were excluded from this analysis.

as frequently as in wild-type cells, suggesting that overexpression of wild-type XRCC4 facilitates homology-independent joining.

One possible mechanism by which XRCC4 might function in end joining was suggested by the observation that XRCC4 interacts with DNA ligase IV (34,35). Plasmid rejoining in ligase IV-deficient yeast is severely impaired (52,53). The predominance of junctions containing short sequence homologies in XRCC4-deficient cells is consistent with the possibility that XRCC4 may function to

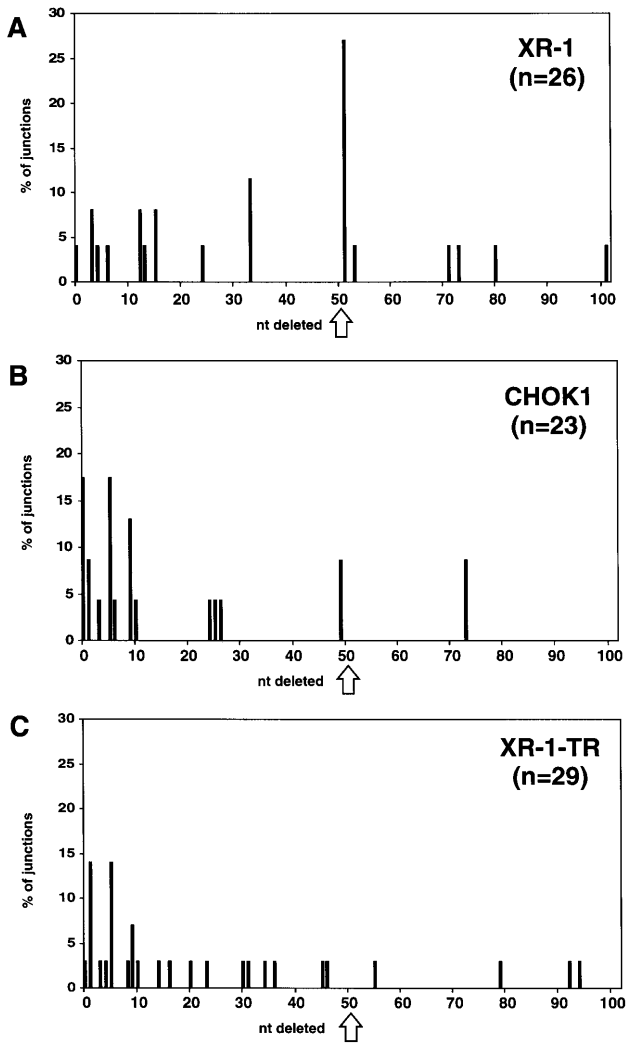

Figure 8. Distribution of deletions at junctions derived from *Cla*I ends. Each histogram shows the distribution of deletion sizes observed in the indicated cell line. The number of junctions analyzed (n) is shown. Data are from Figures 6 and 7. The open arrow indicates the position of the junction corresponding to the 7 nt homology (deletion of 51 nt).

recruit ligase IV to the ends. However, the possibility exists that XRCC4 may play additional roles, such as serving directly as an alignment factor. Also, there are important differences between the results from XRCC4-deficient cells and ligase IV-deficient (53) or LIF1-deficient (54) yeast (LIF-1 is the yeast homolog of XRCC4). Our results show that, unlike the situation in these mutant yeast strains, the overall efficiency of joining in XRCC4-deficient cells is not impaired and sequence homology is not absolutely required, indicating that mammalian cells possess alternative joining mechanisms that can bypass the requirement for XRCC4.

As we observed with XRCC4-deficient cells, the effect of Ku86 deficiency on end joining in mammalian cells is much less severe than in yeast. Rejoining of linearized plasmids in *S.cerevisiae* strains bearing mutations in the genes encoding Ku86 or Ku70 homologs is severely impaired and always proceeds through short sequence homologies (38,39). In xrs-6 cells, the frequency of joining is not decreased and there is only a modest increase in the proportion of junctions with short sequence homologies compared with control cells. These data suggest that mammalian cells possess Ku-independent joining mechanisms that can operate quite efficiently for plasmid rejoining, although not for V(D)J recombination. In this regard, it should be noted that recent biochemical studies have shown that rodent cells possess robust DNA-PK-independent end joining activities (55).

How might Ku86 and XRCC4 align or stabilize end joining intermediates? These proteins could act directly as alignment factors or indirectly by recruitment of other proteins, such as DNA ligases, to the broken ends. The notion that the Ku heterodimer might play an indirect role in recruitment of additional joining activities is supported by several observations. First, biochemical evidence shows that Ku physically interacts with and recruits the catalytic subunit of DNA-PK to sites of DNA damage (15). Second, Ku can act independently of DNA-PK to stimulate end joining by mammalian ligases (56). Recent results have suggested that terminal deoxynucleotidyl transferase may be recruited to DNA ends through interactions with Ku (50). Finally, there is evidence that XRCC4 forms a stable complex with DNA-PK *in vitro* (57). We suggest that Ku and XRCC4 play important roles in the assembly of DNA–protein complexes at sites of DNA damage which help orchestrate the joining of broken ends. This view is consistent with the proposed role for Ku in remodeling of stable DNA–protein complexes formed during the cleavage step of V(D)J recombination (32).

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