MOLECULAR AND CELLULAR BIOLOGY, Oct. 1992, p. 4758–4768
0270-7306/92/104758-11$02.00/0

Vol. 12, No. 10

Analysis of the Defect in DNA End Joining in the Murine *scid* Mutation

JOHN HARRINGTON,[1] CHIH-LIN HSIEH,[1] JENNIFER GERTON,[1] GAYLE BOSMA,*[2] AND MICHAEL R. LIEBER[1]*

Laboratory of Experimental Oncology, Department of Pathology, Stanford University School of Medicine, Stanford, California 94305-5324,[1] and Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111[2]

Received 11 June 1992/Accepted 30 June 1992

Murine severe combined immune deficiency (scid) is marked by a 5,000-fold reduction in coding joint formation in V(D)J recombination of antigen receptors. Others have demonstrated a sensitivity to double-strand breaks generated by ionizing radiation and bleomycin. We were interested in establishing the extent of the defect in intramolecular and intermolecular DNA end joining in lymphoid and nonlymphoid cells from scid mice. We conducted a series of studies probing the ability of these cells to resolve free ends of linear DNA molecules having various biochemical end configurations. We find that the stable integration of linear DNA into scid fibroblasts is reduced 11- to 75-fold compared with that in normal fibroblasts. In contrast, intramolecular and intermolecular end joining occur at normal frequencies in scid lymphocytes and fibroblasts. This normal level of end joining is observed regardless of the type of overhang and regardless of the requirement for nucleolytic activities prior to ligation. The fact that free ends having a wide variety of end configurations are recircularized normally in scid cells rules out certain models for the defect in scid. We discuss the types of DNA end joining reactions that are and are not affected in this double-strand break repair defect in the context of a hairpin model for V(D)J recombination.

The process of DNA end joining in higher eukaryotes is not well understood. Along with the need for a ligating activity, there are indications of the need for an aligning protein in the joining of some but not all DNA end configurations (29). In addition, the relative efficiency of DNA end joining versus homologous recombination varies over 1,000-fold between yeast and mammalian cells (25). The basis for such large variations in the relative levels of end joining is unclear. Previous analyses have been done on the recircularization of linear DNA in simian fibroblasts and have indicated that homologies of a few base pairs are used to align the DNA ends in about 60% of cases but not in the remaining 40% of cases (24).

Thus far, in higher eukaryotes, the only two physiologic site-directed recombination reactions identified are both in the immune system of vertebrates. The V(D)J recombination reaction is a truly site-specific recombination system, whereas immunoglobulin class switch rearrangement is a regionally specific recombination reaction. V(D)J recombination is responsible for the assembly of the complete variable domain exon from subexons called variable (V), diversity (D), or joining (J) elements. A signal sequence, consisting of a palindromic heptamer and an A/T-rich nonamer, lies adjacent to each of the subexonic elements. The V(D)J recombination reaction is directed by a pair of these joining signals—one with a 12-base spacer (12-signal) between the heptamer and nonamer and the other with a 23-base spacer between them (23-signal). Though we do not know a great deal about the mechanism of the reaction yet, it appears that cuts are made at the ends of each signal directly adjacent to the heptamer. The two signal ends are joined together to form a signal joint; the sequences of each signal are precisely retained in over 99% of signal joints (17). The two coding ends undergo nucleotide excision and addition and are then joined to form a coding joint. Hence, unlike the precise signal joint, the coding joint is characteristically imprecise with respect to the nucleotide sequence at the junction. This imprecision is important for generating binding diversity. These two halves of the reaction—signal joint and coding joint formation—are dissociated in the murine defect called scid (severe combined immune deficiency).

Relevant to both site-directed recombination and double-strand break repair is the murine *scid* mutation (26). Mice homozygous for this mutation, scid mice, fail to make antigen receptors (2). Instead of assembled variable domain exons at the immunoglobulin and T-cell receptor loci, deletions of hundreds to thousands of base pairs are found around the areas where V(D)J recombination should have occurred (5, 10, 20, 22, 23, 27, 28). Analysis of pre-B and pre-T cells from these mice has shown that the reaction initiates and that the recombination signals are joined (Fig. 1A), but that resolution of the attached coding segments is reduced at least 100-fold (Fig. 1B) (18).

There may be a relationship between the DNA enzymology for resolving coding ends in the site-directed V(D)J recombination reaction and that for general double-strand break repair. This is indicated by the observation that scid fibroblasts show increased sensitivity relative to normals when exposed to ionizing radiation and other free radical-generating agents that cause double-strand breaks (1a, 3, 6).

We are interested in the genetic and biochemical nature of the defect in scid mice and how the *scid* gene product functions in coding end resolution of V(D)J recombination and in double-strand break repair. As part of our analysis of this defect, we have conducted a study of DNA end joining in scid cells. We find that some types of DNA end joining are affected and others are not.

* Corresponding author.

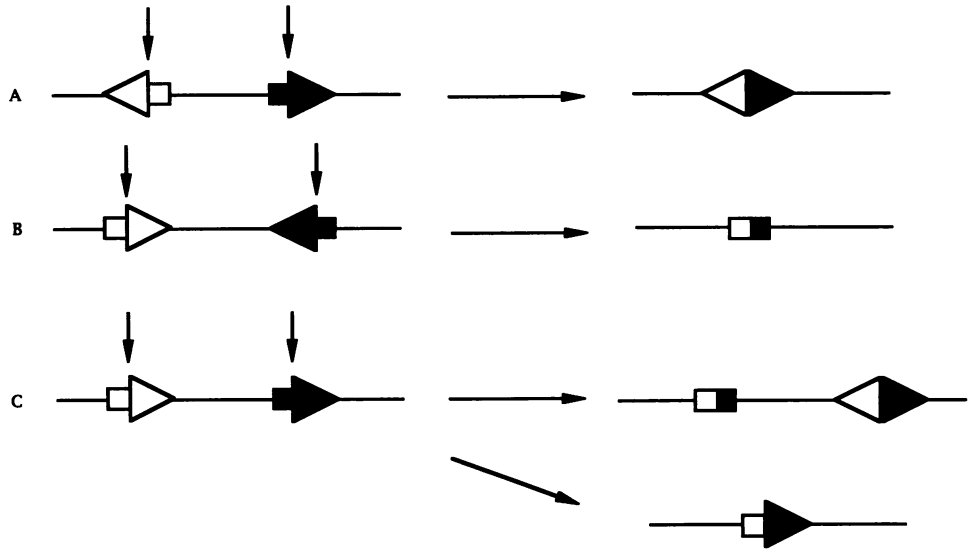

FIG. 1. Reactions tested by V(D)J recombination substrates. The recombination zone of each of three V(D)J recombination substrates is shown. The substrates contain a given recombination zone positioned within a plasmid at the site of linearization shown in Fig. 3. The signal sequences for this site-specific reaction are depicted as triangles. A transcription terminator is positioned between the signals. The two different recombination signals are symbolized as open (12-base spacer) or filled (23-base spacer) triangles. The vertical arrows are the sites of crossover. In line C, an inversion reaction is depicted (top of Fig. 1C). An inversion can also yield a hybrid joint outcome (bottom of Fig. 1C). In lines A and B, deletional reactions are shown in which either the signal or coding joint is retained, respectively. Line A corresponds to the substrate pJH200. Line B corresponds to the substrate pJH290-2. Line C corresponds to the substrate pJH299.

MATERIALS AND METHODS

Plasmids and their use in end joining assays. All of the plasmids used here have been described previously (7, 8, 13, 19) except for pML142N and pRSVlacZNeo. pRSVlacZNeo is derived from pAcLac (9) by insertion of the Rous sarcoma virus long terminal repeat at the *Bam*HI site (destroyed by Klenow fill-in and ligation) and by insertion of the neomycin transcription unit contained in the *Eco*RI to *Bam*HI fragment of pdBPV-MMTNeo(342-12) (13) into the *Xba*I site of pAcLac by using *Xba*I linkers. This leaves the *neo* gene in the same orientation as the *lacZ* gene and directly 3′ of it. The neomycin transcription unit derived from pdBPV-MMTneo utilizes the mouse metallothionein I promoter and the simian virus 40 polyadenylation and splice site fragment (13). For the DNA integration studies, the pRSVlacZNeo was linearized at the unique *Xmn*I site, which is located within the β-lactamase (ampicillin resistance) gene.

pML142N has a prokaryotic transcription terminator positioned between a prokaryotic promoter and the gene for chloramphenicol acetyltransferase (*cat*), similar to other plasmids that we have described previously (7, 18). Linearization of this plasmid with *Sal*I and *Bam*HI cuts out the transcription terminator. In the recircularization (intramolecular end joining) assays, this linearized plasmid is transfected into eukaryotic cells. Some fraction of the transfected DNA is recircularized in the eukaryotic cells. Recircularization of this molecule now allows the promoter to direct transcripts into the *cat* gene without termination. Hence, recircularization products of this linearized plasmid are ampicillin and chloramphenicol resistant. In the recircularization assays, transformation of the linearized DNA showed that less than one part in 10^5 was left uncut. Moreover, any uncut molecules contaminating the initial preparation of linear DNA will not be detected in the recircularization assays because they will retain the transcription terminator that normally resides between the *Sal*I and *Bam*HI sites.

Cell lines. The lymphoid cell lines from normal (19) and scid mice (18) have been described previously. The normal (NF-22) and scid (SF-7 and SF-19) fibroblasts were derived from day 18 explants of C.B-17 embryos and isolated by a passage and cell density 3T3 protocol (30). The three fibroblast lines are within 36 passages of their inception.

Cell culture. The normal fibroblasts (NF-22) and scid fibroblasts (SF-7 and SF-19) are grown in RPMI 1640 media with 10% fetal calf serum and penicillin and streptomycin. The culturing of the lymphoid cells has been described (7, 19).

Transfection and recovery of the DNA. Except in the DNA integration study, the lymphocytes and fibroblasts are transfected with a hypotonic variant of the DEAE-dextran protocol, as described previously (19). Recovery of the plasmid DNA is performed by using an alkaline lysis procedure, as described previously (7).

For DNA integration into fibroblasts, electroporation and calcium phosphate methods were used. In the electroporation method, between 3×10^6 and 10×10^6 cells (50% confluence) are removed with trypsin-EDTA solutions and washed once with RPMI 1640–10% fetal calf serum-penicillin or streptomycin. The cells are then washed once with serum-free RPMI 1640 and resuspended in 0.8 ml of RPMI 1640 containing 10 μg of the DNA to be transfected. The voltage is 240 V, the capacitance is 960 μF, and the gap distance in the chamber is 0.4 cm. All of these manipulations are done at room temperature. Immediately after electroporation, the cells are transferred back to complete media and cultured.

In the calcium phosphate transfection of fibroblasts, the procedure is as follows. A 2× HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) solution consisting of

8 g of NaCl, 0.105 g of $NaHPO_4$, and 6.6 g of HEPES in H_2O is prepared to a total solution volume of 0.5 liters after adjusting the pH to 7.0. A 0.5-ml DNA solution is prepared by adding 0.061 ml of 2 M $CaCl_2$, 0.434 ml of H_2O, and 0.005 ml of DNA at 1 μg/ml in that order to a 1.5-ml tube. The DNA solution is added dropwise to 0.5 ml of the 2× HEPES solution in a 1.5-ml tube, all at room temperature (approximately 22°C). The solution is not mixed and is vaguely cloudy at this point. This transfection solution is immediately sprinkled onto the tissue culture cells by using a Gilson Pipetman. The dish is immediately placed into the 37°C incubator. For a 100-mm culture dish, 1 ml of transfection solution is used. The cells are washed once with phosphate-buffered saline at 16 h, and medium is added.

V(D)J recombination assay. The assay for V(D)J recombination has been described previously (7, 14, 17, 18) and more recently, with modifications in the transformation step (8), but is only described briefly here. The plasmid substrates that are used in this assay have recombination zones that are diagrammed in Fig. 1. The orientation of the heptamer-nonamer V(D)J recombination signals (depicted as triangles in Fig. 1) on the substrates depict the outcome of the reaction. The basic principle of these substrates is the same. A prokaryotic transcription terminator is located between the two recombination signals in the recombination zone. Deletion of this terminator in orientations in Fig. 1A or B, or inversion of this terminator in the orientation in Fig. 1C, allows transcripts from a prokaryotic promoter to proceed into an antibiotic resistance gene and allow its expression. The recombination zones depicted in Fig. 1 are part of plasmids in which the surrounding DNA is structured like pML142N. Outside of the recombination zones, the plasmids pJH299 and pJH290-2 are identical and pJH200 is similar to the pML142N. Within the recombination zones, pJH299 has signals arranged for inversion (Fig. 1C) and pJH290-2 has signals arranged for deletion (Fig. 1B).

The V(D)J recombination substrates are used in the assay of V(D)J recombination activity levels as follows (see references 7 and 17–19 for details). The substrates are transfected into lymphoid cells, whereupon they replicate because they carry the polyoma origin and large T coding sequences. Replication can be used to document DNA entry and assess what fraction of the recovered plasmid population was eligible for recombination (see Results). After 48 h in the lymphoid cells, the plasmids are harvested and analyzed for recombination by transformation into *Escherichia coli*. Plasmids that have undergone V(D)J recombination confer resistance to both ampicillin and chloramphenicol, whereas both substrate and product plasmids confer resistance to ampicillin. Therefore, the ratio of the number of colonies on the ampicillin-chloramphenicol (AC) selection plates to the number on the ampicillin selection plates (A) reflects the fraction (expressed as a percentage) of the recovered DNA molecules that recombined during the time in the lymphoid cells.

Use of DNA replication for assessing transfection efficiency and DNA entry. Plasmids that bear a polyoma origin of replication will replicate in the murine lymphoid cells if and only if polyoma T antigen is present. In such cases, the plasmids that enter the cells efficiently replicate and V(D)J recombinants are almost exclusively detected in the replicated pool of molecules (1a). Molecules that failed to enter the cells or are adherent to the outside of the cells will not replicate or undergo V(D)J recombination. Therefore, DNA replication does not significantly over- or underestimate the pool of molecules that entered the cells.

We distinguish the pool of plasmids that have replicated in the eukaryotic cells by virtue of the fact that they have lost their prokaryotic *dam* methylation at the A in GATC sites. *Dpn*I is a restriction enzyme which eliminates plasmid molecules that did not replicate and, therefore, retain the *dam* methylation. We designate treatment with *Dpn*I with a D. Hence, transformants arising on ampicillin plates from DNA that has been treated with *Dpn*I is designated DNA. The percentage of a population of recovered plasmids that has replicated is 100 × (DA/A).

Intermolecular end joining assay. The joining of two linearized plasmids, pJH104 bearing ampicillin resistance (A) and pACYC184 bearing tetracycline and chloramphenicol resistance (C), was examined in this study. The plasmid pJH104 also bears the polyoma early region and a ColE1 prokaryotic origin (1a). The plasmid pACYC184 also bears the prokaryotic p15A origin. The plasmid pACYC184 cannot replicate in eukaryotic cells because it does not bear a eukaryotic origin of replication. Only after recombination with the polyoma origin bearing the AlwNI fragment of pJH104 can the chloramphenicol-bearing linearized pACYC184 undergo replication. In the transfection of 3 × 10^6 lymphoid cells, 150 ng of the linearized pACYC184, 24 ng of the linearized plasmid pJH104, and 150 ng of supercoiled pCLH11 were cotransfected. The pCLH11 plasmid bears the kanamycin resistance gene (designated K), the polyoma early region, and the prokaryotic ColE1 origin. The pCLH11 plasmid replicates autonomously and permits normalization of transfection. The replication of pCLH11 is given as DK/K, which is the ratio of the number of *Dpn*I-resistant kanamycin (DK) colonies divided by the total number of kanamycin (K) colonies. The ratio AC/DK is the intermolecular recombination frequency normalized for transfection.

Several controls indicate that the AC colonies arise from intermolecular recombination rather than from the cotransformation of individual *E. coli* cells by two separate plasmids. First, DNA prepared from AC colonies was analyzed as uncut DNA and as digested DNA by using the following restriction enzyme combinations: *Sma*I, *Cla*I × *Sma*I double digest, *Cla*I × *Dra*I double digest, and *Stu*I. The undigested DNA demonstrated the presence of only one plasmid in each case, and these plasmids were larger than either pJH104 or pACYC184 alone. The restriction analyses of these recombinant plasmids demonstrated that the sum of their fragments added up to the size of the undigested DNA. Second, the AC colony frequencies were undiminished by *Dpn*I digestion. Recircularized pACYC184 cannot replicate in eukaryotic cells because it bears no origin. Hence, double transformants with pACYC184 would be eliminated by *Dpn*I digestion prior to transformation. Third, the double transformation frequency of the two linearized plasmids directly into *E. coli* was undetectable and was at least 1,000-fold less than the frequency of AC colonies. Fourth, the transfection of each linear plasmid individually into the lymphoid cells followed by pooling of the recovered DNA did not give rise to double transformants and was at least 1,000-fold less than the frequency of AC colonies. Each transfection was analyzed by pooling the results from multiple transformations of *E. coli*.

RESULTS

The level of the residual coding joint resolution activity in the scid lymphoid V(D)J recombination reaction. In analyzing the various types of end joining reactions in normal and scid cells, we were interested in determining the magnitude of the

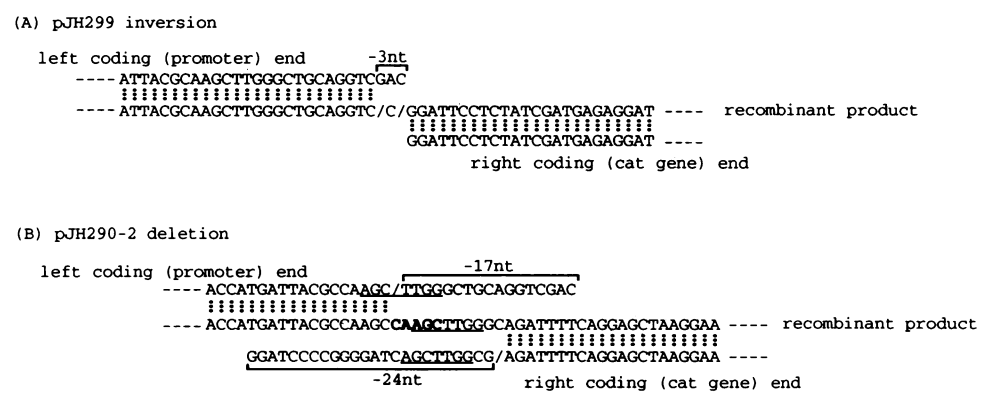

FIG. 2. The sequences of V(D)J recombination coding end resolution products from scid lymphoid cells. One strand of the coding joint from an inversional recombination event is shown in the middle line of panel A. The three dots between identical bases indicate the portions of the two participating coding ends that contributed to the recombinant product; only one strand of each coding end is shown. The slashes indicate the deletion endpoints within each coding end. Only 3 bp (GAC) were deleted from the left (promoter) coding end (formerly attached to the 12-spacer signal). The C between the slashes is a single-nucleotide addition between the two recombinant coding ends. No nucleotides were lost from the right (*cat* gene) coding end, which was formerly attached to the 23-spacer signal. (B) Symbols are the same as in panel A. One strand of the recombinant product is shown in the middle, and the corresponding portions of the contributing coding ends are shown above and below it. Seventeen nucleotides were lost from the left coding end. Twenty-four nucleotides were lost from the right coding end. There is evidence of considerable processing in the 11 nucleotides that are not directly contiguous with either end. There is a 7-nucleotide region of homology between the two participating coding ends which is underlined in the two coding ends and in the product. There is a 5-nucleotide direct duplication in the product (indicated by bold letters) that is not present in either participating coding end. The repeat duplicates the 5 nucleotides immediately to the left of itself in the left (promoter) coding end. The two nucleotides (GC) to the right of the underlined region are present in the left coding end and the product but not in the right coding end. A slip-mispair priming event (24) at the left coding end would explain these features of the left end. The joining of this processed left end with the right coding end could occur in a variety of ways that may or may not be similar to coding end joining in V(D)J recombination.

deficit in the site-specific V(D)J recombination reaction. In a previous analysis (18), we detected no coding joint formation activity except when we provided 70-bp blocks of homology at the coding ends. However, we only screened for recombination down to a level of approximately 0.005 coding joints per signal joint formed. In order to compare coding end resolution in scid with end joining in scid, we needed to know whether there was any residual V(D)J recombination coding end resolution in scid lymphoid cells at a level at least 10-fold lower than we had previously determined. We have primarily used the inversion substrate pJH299 for this study. The advantage of using inversion substrates is that they undergo V(D)J recombination to yield products that bear both the precise signal and the imprecise coding joints (top line of Fig. 1C) (17). Analysis of the signal joint from an inversion documents that the rearrangement was a V(D)J recombination event. Results from substrates that only undergo deletional V(D)J recombination to retain the imprecise, coding joint half of the reaction (Fig. 1B) are more difficult to interpret because the rare coding joints that form may have been generated by illegitimate recombination that deleted at approximately the correct sites in the substrate. Deletion substrates that retain the precise signal joint half of the V(D)J recombination reaction (Fig. 1A) are readily documented as V(D)J recombination events. By comparing the ratio of the frequency of signal joint formation (by using a signal joint deletion substrate) to inversional recombination (which requires both signal and coding joint formation), the magnitude of the deficit in coding joint formation can be determined. The recombination regions diagrammed in Fig. 1 register as recombinant products in an assay that has been described previously (7, 17–19) and is described in Materials and Methods.

We have compared the recombination of the inversion substrate, pJH299 (Fig. 1C), to one that retains only the signal joint, pJH200 (Fig. 1A). In cells from wild-type mice, the recombination frequencies of these two substrates are usually within 2.5-fold of each other (17, 18). Specifically, in the studies here, the recombination frequency of pJH200, the signal joint retaining substrate, was 4% in a wild-type pre-B-cell line called 1-8. That is, 4% of the recovered plasmid had undergone V(D)J recombination to generate signal joints; the remaining 96% of the analyzed plasmids had entered the cells (see Materials and Methods for DNA entry determination) but had not undergone recombination. The recombination frequency of the inversion substrate, pJH299, was 1.7% in this line. The scid pre-B-cell line, S55, generates signal joints (i.e., recombines pJH200) at a frequency of 0.5%. However, the recombination frequency with the inversion substrate, pJH299, was only 0.01% (as determined from analysis of 5.8×10^5 replicated plasmids). Restriction analysis showed that 57 of 58 of these recombinants were hybrid joints (Fig. 1C). One of seven hybrid joints sequenced showed a 4-bp inverted repeat addition (GTCG) at a full-length left coding end of pJH299 (substrate coding end shown in Fig. 2A); the others were similar to scid hybrid joints described previously (18). Only 1 of the 58 recombinants proved to be an inversion, which was sequenced. The sequence of the coding joint was normal (Fig. 2A); the sequence of the signal joint demonstrated a precise heptamer to heptamer join as occurs in 50% of signal joints in scid (17, 18). The corrected inversional recombination frequency was 1 of 584,000 or 0.0002%. Acknowledging the limitations due to such a low frequency of inversion events, one, nevertheless, can estimate that coding joint formation is reduced by the ratio of the signal joint formation divided by the inversion frequency (0.5% divided by 0.0002%) or 2,500-fold. In a similar series of scid lymphoid cell transfections with the same substrate and analyzing 6×10^5 replicated plasmids, we found no inversions, indicating that the coding joint forming capability may be reduced approximately 5,000-fold. A 5,000-fold reduction in coding joints relative to

TABLE 1. Integration of linear DNA into scid fibroblasts is reduced

Transfection method	Interval (h) between transfection and start of G418	Cell line	No. of cells (10^6)	No. of resistant clones	Dilution	Stable transfection frequency
Electroporation	24	NF22	3	902	1:1	3.0×10^{-4}
	24	NF22	3	1,240	1:1	4.1×10^{-4}
	24	SF7	3	15	1:1	5.0×10^{-6}
	24	SF7	3	13	1:1	4.3×10^{-6}
Electroporation	48	NF22	8	77	1:100	9.6×10^{-4}
	48	SF7	8	3	1:100	3.8×10^{-5}
	48	SF19	8	7	1:100	8.8×10^{-5}
Calcium phosphate	48	NF22	3.6	41	1:1	1.1×10^{-5}
	48	SF19	3.6	3	1:1	8.3×10^{-7}

[a] Fibroblasts (50% confluence) were transfected with the linearized neomycin expression vector, pRSVlacZNeo. After dilution and plating, the cells were placed under selection for G418 resistance (400 μg/ml) beginning 24 or 48 hrs after transfection, were harvested 14 days after transfection, and were examined for the number of resistant colonies.

signal joints corresponds to fewer than 0.0002 coding joints for every signal joint that forms in scid lymphoid cells. In contrast, in lymphoid cells from normal mice, the frequency is between 0.4 and 1 coding joints for every signal joint, as determined by using these same substrates (18).

We also compared the frequency of recombinants from a coding joint-retaining deletion substrate, called pJH290-2 (recombination zone as in Fig. 1B), with the frequency of signal joint formation on pJH200 (Fig. 1A). We detected one pJH290-2 recombinant of 72,000 substrates that had entered the scid lymphoid cells. This one recombinant of pJH290-2 (Fig. 2B) had markedly greater nucleotide loss from each coding end than is normally observed in V(D)J recombination. The sequence departs from the substrate beginning 17 bp recessed from one coding end and 24 bp recessed from the other. Interestingly, the resolution involved a 7-bp stretch of homology shared between the two coding ends (Fig. 2B). In addition, the area of new synthesis between the recombinant ends is consistent with the type of strand slippage that has previously been described to occur at DNA end joining events in nonlymphoid cells (24). The total loss of 41 bp (17 plus 24 bp) of sequence from the original coding ends is sufficiently atypical of V(D)J coding end resolution (17) that it suggests that this junction was formed by general cellular end joining activities after the coding ends were released from an aborted V(D)J recombination reaction. Therefore, we do not consider this latter junction to be an indication of residual V(D)J recombination activity. Because we did not detect any coding joints that appeared to be resolved as normal V(D)J recombination events, the determination of the deficit in coding joint formation in this study can only be a lower estimate. The ratio of signal to coding joint formation in this analysis is more than 360-fold (0.5% divided by 0.0014%). These results are consistent with the larger deficit in coding joint formation indicated above by the signal joint-to-inversion ratios.

Integration of linear DNA into the genome of scid fibroblasts is markedly reduced. It has been shown that double-strand break repair is deficient in scid fibroblasts and lymphocytes (1, 6). We were interested in whether this defect in DNA end joining for murine scid would affect the stable integration of linear DNA into the cells. To test this, we linearized a eukaryotic neomycin resistance vector, pRSVlacZNeo (see Materials and Methods) and transfected normal or scid fibroblasts with it by electroporation and calcium phosphate methods. The transfected fibroblasts were placed under G418 selection for 14 days. We examined the stable transfection efficiency in experiments in which we began the drug selection at both 24 and 48 h after transfection to be certain that any differences were not simply due to rates of expression. We previously established that the survival curves for the normal and scid cells were indistinguishable as a function of G418 concentration and that the plating efficiencies of successfully transfected normal and scid cells were the same at the G418 concentration (400 μg/ml) used in the selections (data not shown). During the period of selection, colonies of G418 resistant cells grew out. At the end of the 14 days, the resistant clones were counted. Transient transfections with the same DNA concentration of supercoiled luciferase expression vector, pCLH12, were done by the same transfection methods in order to assess for any differences in the efficiency of DNA uptake. The cells transfected with pCLH12 were harvested 48 h after transfection and analyzed for luciferase activity. The normal and scid fibroblasts were found to express at very similar levels (within a factor of 1.4) regardless of whether electroporation or calcium phosphate methods were used. The transfection efficiencies were higher by electroporation, however. Parallel, control transfections with the same concentrations of sheared salmon sperm DNA instead of linearized pRSVlacZNeo yielded no stably G418-resistant fibroblast colonies, indicating that there was no breakthrough in the selection.

Despite beginning with equal numbers of cells and very similar DNA entry and expression efficiencies on the basis of the luciferase assay, there was an 11- to 75-fold reduction in the frequency of stable integration in the scid cells (Table 1). The reduction is large regardless of the time interval between transfection and selection, and the reduction exists for each of the two scid fibroblast lines relative to the normal line from the same murine genetic background (C.B-17). Hence, the integration of linear DNA into the genome of murine scid fibroblasts appears to be reduced compared with normal fibroblasts.

Analysis of intramolecular end joining (recircularization) in normal and scid fibroblasts. It has been shown previously that linearized plasmids are no less efficiently recircularized in scid than in normal lymphoid cells (18). However, in this earlier analysis, only compatible 5′ overhangs generated by *Bam*HI were examined. We considered that testing of incompatible ends may more accurately reflect the end joining process in coding joint formation, ionizing radiation induced double-strand break repair, and integration of DNA. Specif-

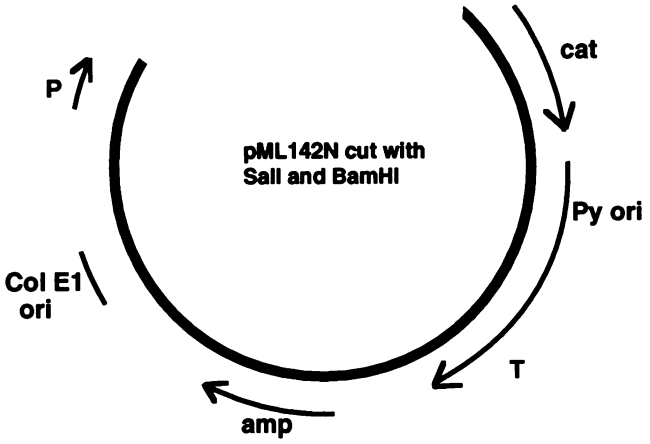

FIG. 3. Linearized pML142N, the substrate for recircularization. Beginning from the prokaryotic promoter (P) and moving clockwise, the molecule bears the chloramphenicol acetyltransferase gene (*cat*), the early region of polyoma (containing the late promoter, the enhancer, the polyoma origin, the early promoter, and the T antigen genes), the β-lactamase gene, and the prokaryotic origin of replication. Recircularization of the molecule results in the prokaryotic promoter directing transcripts into the *cat* gene. The prokaryotic promoter is 83 bp from its proximate DNA end, and the *cat* gene begins 50 bp from its proximate DNA end.

ically, are ends that do not share compatible overhangs, or that require some DNA repair, joined as efficiently in scid as in normal cells?

For these studies, we digested a plasmid, pML142N, with the restriction enzymes *Sal*I and *Bam*HI (Fig. 3). This digestion liberates a fragment containing the transcriptional terminator and recombination signals that are present on this plasmid. The resulting linear molecule of 7,700 bp has incompatible 5′ overhanging *Sal*I and *Bam*HI ends (Fig. 4A). We documented completion of digestion by transformation of the linear preparation of DNA into *Escherichia coli*. This showed that residual undigested plasmid was less than one

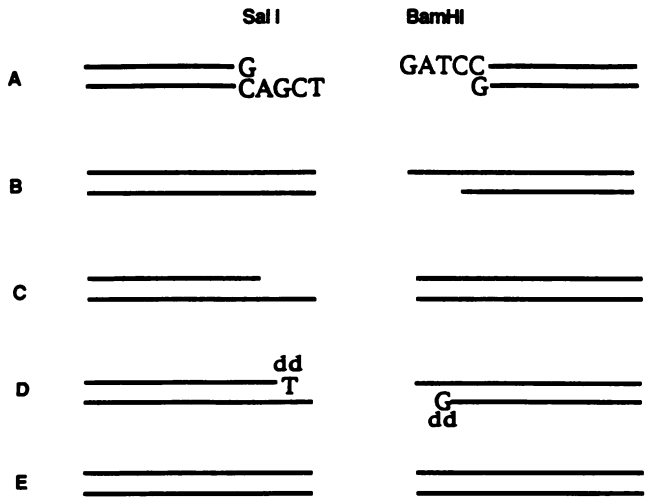

FIG. 4. DNA ends in recircularization end joining experiments. The overhang of each of the DNA ends is shown. The end closest to the prokaryotic promoter (*Sal*I) is shown on the left. The end closest to the *cat* gene (*Bam*HI) is shown on the right.

part in 10^5. We used this DNA to generate the four other combinations of ends shown in Fig. 4B through E. These were *Sal*I filled in (Fig. 4B), *Bam*HI filled in (FIg. 4C), dideoxy T and dideoxy G filled in (Fig. 4D), and *Sal*I and *Bam*HI filled in (Fig. 4E). Resolution of the dideoxy filled-in ends requires the removal of at least these nucleotides prior to resolution. Fibroblasts were transfected with each of these linearized molecules along with an equal amount of the supercoiled polyoma T antigen expression vector, pJH104, for the purpose of normalizing for transfection efficiency based on DNA replication (see Materials and Methods and below). The extrachromosomal DNA was harvested at 48 h after transfection. The DNA was transformed into *E. coli* to detect recovered plasmids. A portion of the transformation was plated onto ampicillin plates (designated A) and a portion onto ampicillin-chloramphenicol (designated AC). Both the recircularized pML142N plasmid and the supercoiled pJH104 plasmid give rise to colonies on the ampicillin (A) plate. Only recircularized pML142N gives rise to colonies on the ampicillin-chloramphenicol plate (AC). We find that the number of AC colonies is usually between 0.05% and 1% of the number of A colonies harvested from normal fibroblasts. Hence, almost all of the A colonies are due to transformation with pJH104. Moreover, all of the plasmids giving rise to AC colonies can be destroyed by *Bgl*II, which only cuts the recircularized pML142N. Therefore, the ratio AC/A reflects the number of linear pML142N molecules that recircularize divided by the number of pJH104.

Both pJH104 and pML142N bear polyoma origins of replication and polyoma T antigen coding sequences. Therefore, they can replicate in the eukaryotic cells. We have shown previously that replication of polyoma origin bearing plasmids can be used as a marker for plasmid entry (see Materials and Methods) (19). That is, replication neither over- nor underestimates DNA entry. As the plasmids replicate, they lose their adenine methylation at the GATC sites and become resistant to the restriction enzyme *Dpn*I. Here, we analyzed the eukaryotic DNA harvest for AC and A after *Dpn*I digestion. We find that the number of AC colonies is not reduced by the *Dpn*I digestion. This indicates that linear pML142N molecules are efficiently replicated after they recircularize. We have previously found that essentially all circular DNA bearing the polyoma early region replicates efficiently after entry into murine cells (19). Transformation frequencies based on *Dpn*I-treated DNA are designated DA instead of A. The ratio DA/A is the fraction of pJH104 that replicated and reflects the transfection efficiency. On the basis of this, the ratio AC/DA reflects the frequency of recircularization of linear pML142N molecules normalized for the replication of the cotransfected circular plasmid (pJH104). This ratio is likely to be an underestimate of the true recircularization efficiency because replication is likely to be less efficient until recircularization has occurred. We are normalizing for transfection by using a plasmid, pJH104 (the denominator of the AC/DA ratio), that is already circular upon entry, and the propagation of pJH104 can only be equal to or greater than the propagation of the linearized pML142 molecule that must first recircularize before efficient propagation can begin (the numerator of the AC/DA ratio). For these reasons, the recircularization ratio is the lower limit for the efficiency of recircularization.

In normal fibroblasts the recircularization ratio is usually between 0.1×10^{-2} and 0.8×10^{-2} regardless of the end combination involved (Table 2). Even the dideoxynucleotide filled-in ends join no less efficiently than the other end combinations. A repeat of the same analysis in scid fibro-

TABLE 2. Intramolecular end joining in normal and scid fibroblasts[a]

Phenotype and type of end	Transformants			
	AC	A (10^3)	DA/A	AC/DA (10^{-2})
Normal				
A	26	24	0.29	**0.39**
	61	49	0.79	**0.15**
B	1*	18	0.52	**0.01**
	19	35	0.22	**0.25**
C	115	42	0.35	**0.81**
	60	52	0.23	**0.51**
D	18	12	0.40	**0.38**
	110	57	0.42	**0.45**
E	98	27	0.38	**0.95**
	45	6.3	0.40	**1.8**
scid				
A	20	14	0.99	**0.15**
	80	18	0.59	**0.75**
B	77	17	0.58	**0.77**
	13	16	0.58	**0.14**
C	12	19	0.17	**0.36**
	255	41	0.47	**1.3**
D	5*	23	0.90	**0.02**
	119	33	0.57	**0.63**
E	66	22	0.23	**1.3**
	19	23	0.67	**0.12**

[a] Fibroblasts (50% confluence; 10^7 total number of cells) were transfected with 150 ng of linearized pML142N and 150 ng of pJH104. The DNA was harvested 48 h after transfection and analyzed for DNA replication (DA) and the number of transformants with ampicillin (A) or ampicillin-chloramphenicol (AC) resistance. AC/DA is the ratio of recircularized pML142N to the number of replicated pJH104 plasmids. *, transfections that resulted in low numbers of recombinants; these transfections may yield less reliable measures of the recircularization frequency.

TABLE 3. Intramolecular end joining in normal and scid lymphocytes[a]

Phenotype and type of end	Transformants			
	AC	A (10^3)	DA/A	AC/DA (10^{-2})
Normal				
A	119	60	1.0	**0.20**
	591	95	0.80	**0.78**
	2	8	0.07	**0.31**
B	107	76	1.0	**0.14**
	9	9	0.29	**0.31**
D	81	63	0.88	**0.15**
	47	63	0.95	**0.08**
	176	74	0.90	**0.27**
E	33	38	1.0	**0.09**
	175	76	1.0	**0.23**
	59	20	0.55	**0.55**
scid				
A	27	1,031	0.041	**0.064**
B	22	1,490	0.023	**0.064**
D	7	1,400	0.016	**0.031**
E	27	792	0.063	**0.054**

[a] Designations are as described in the footnote to Table 2. The DNA end configuration C was not tested.

blasts showed that they recircularized the various linearized substrates no less efficiently than normal fibroblasts regardless of the end configuration (Table 2). The only type of end not listed in Table 2 is a 3′ overhang. In separate studies with another substrate in which a linear molecule had one 3′ overhang and one blunt end, the recircularization frequency was similar to those listed and there was no difference between normal and scid fibroblasts (data not shown). We conclude that DNA end joining of a variety of combinations of incompatible ends is normal in scid fibroblasts.

Analysis of intramolecular end joining (recircularization) in normal and scid lymphocytes. We were interested in whether lymphocytes would demonstrate any differences from fibroblasts in end joining. Therefore, we repeated the above determinations of recircularization frequency in normal and scid pre-B lymphoid cell lines. Recircularization of pML142N in normal lymphocytes was as efficient as in normal fibroblasts (Table 3). The recircularization efficiency of scid lymphocytes was two- to fivefold lower on average than the recircularization in normal lymphocytes (Table 3). However, the type of overhang or end configuration did not influence the recircularization efficiency in scid lymphocytes, and these small differences were not observed in another end joining assay described below. Overall, neither the *scid* mutation nor the lineage difference (lymphocytes versus fibroblasts) markedly affected the intramolecular recircularization efficiency.

Analysis of intermolecular end joining in normal and scid lymphocytes. In evaluating the large effects of the scid mutation on V(D)J recombination and DNA integration versus the small effect on intramolecular recircularization, we considered the possibility that the manifestation of the effect of the mutation might be more apparent in an assay that tested the intermolecular interaction of DNA ends. The collision frequency of two DNA ends on different molecules is likely to be much lower than that frequency in intramolecular recombination. We devised an assay in which we determined the frequency of intermolecular end joining between two linearized plasmids, one carrying ampicillin (linearized pJH104) and the other carrying chloramphenicol (designated C) and tetracycline resistance markers (linearized pACYC184) (Fig. 5). In this assay, a circular plasmid is also introduced into the cells, as before, to permit normalization for DNA entry. The circular plasmid, pCLH11, carries only kanamycin resistance (designated K). The intermolecular end joining frequency is determined as AC/K. The ratio, AC/DK, is the number of intermolecular recombination events between the two linearized plasmids divided by the replication of the circular plasmid (Table 4).

Transfection of normal and scid lymphocytes with the two linear molecules and the circular plasmid resulted in a AC/DK ratio of between 2×10^{-5} and 5×10^{-4}. The number of AC and DAC were similar, indicating that most recombinants also undergo subsequent DNA replication. There is no difference between scid and normal in the intermolecular recombination efficiency (Table 4).

The restriction enzyme used to linearize pACYC184 is *Xba*I, which generates a 5′ overhang. The enzyme AlwNI was used to linearize pJH104, and this generates a 3′ overhang. Despite the incompatible ends, restriction enzyme

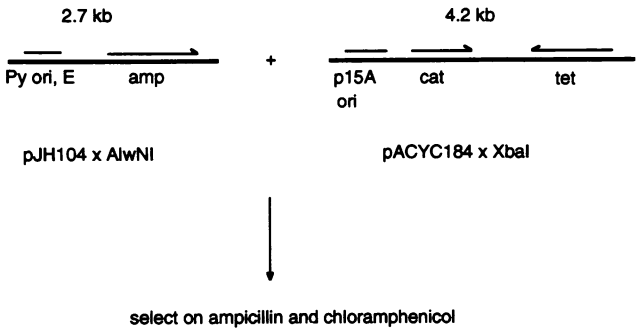

FIG. 5. Intermolecular end joining assay. The molecule on the left is 2.7 kb and bears the polyoma origin and enhancer, and the β-lactamase gene. The molecule on the right is 4.2 kb and bears the p15A prokaryotic origin, the *cat* gene, and the tetracycline gene (*tet*). Plasmids that are recombinant products of these two molecules are selected for by plating the bacterial transformants on ampicillin-chloramphenicol (AC) plates.

analysis on the intermolecular recombinants demonstrated that 50% of 72 events analyzed had recombined within 200 bp of the free end of each linear molecule. Moreover, 35% of the intermolecular recombinants retained the tetracycline resistance gene. The promoter for this gene is 85 bp from one free end. Therefore, in this group, the base loss does not extend more than 85 bp in from this end. There were no differences between normal and scid lymphocytes with respect to the percentage of intermolecular recombinants that retained an intact tetracycline gene.

Nucleotide loss, addition and homology usage in the joining of free ends in normal and scid fibroblasts and lymphocytes. Though the frequency of intra- and intermolecular end joining was similar in normal and scid cells, we considered the possibility that the lengths of deletions or end modifications might be greater in scid. That is, while the quantity of end joining might be normal, the quality of it might not be. We sequenced the junctions in 10 scid and 14 normal fibroblast intramolecular end joining products (Table 5). Comparing these two small samplings, there were no marked differences in the lengths of nucleotide loss at either end.

TABLE 4. Intermolecular end joining in normal and scid lymphocytes[a]

Phenotype	Transformants			
	AC	K (10^{-3})	DK/K	AC/DK (10^{-4})
Normal	22	273	0.57	**1.4**
	2	86	0.14	**1.7**
	4	220	0.18	**1.1**
	3	130	1.0	**0.23**
scid	3	230	0.24	**0.54**
	11	192	0.17	**3.4**
	35	210	0.24	**5.0**
	7	230	0.20	**1.5**

[a] As described in Materials and Methods, the joining of two linearized plasmids, pJH104 bearing ampicillin resistance (A) and pACYC184 bearing tetracycline and chloramphenicol resistance (C), was examined in this study. The pCLH11 plasmid replicates autonomously and permits normalization of transfection. The replication of pCLH11 is given as DK/K, which is the ratio of the number of *Dpn*I-resistant kanamycin (DK) colonies divided by the total number of kanamycin (K) colonies. The ratio AC/DK is the intermolecular recombination frequency normalized for transfection. Each line of the table represents a separate eukaryotic transfection.

TABLE 5. Nucleotide loss and addition in fibroblasts at recircularization junctions

Type of end and phenotype	Nucleotide loss or addition[a]		
	cat gene end	Joint	Promoter end
A			
Normal	**−16** (*n* = 1)	**+42** (*n* = 1)	**−33** (*n* = 2)
	−16	+42	−33
			−33
scid	**−7.3** (*n* = 3)	**+21** (*n* = 3)	**−74** (*n* = 3)
	−4	0	−21
	−5	0	−16
	−13	+62	−184
B			
Normal	**0** (*n* = 1)	**0** (*n* = 1)	**−35**** (*n* = 1)
scid	**−2** (*n* = 2)	**0** (*n* = 2)	**−16.5** (*n* = 2)
	−1	0	0
	−3	0	−33**
C			
Normal	**−1.6** (*n* = 5)	**+23** (*n* = 5)	**−9.6** (*n* = 5)
	0	+115	−42
	−1	0	0
	7	0	−6*
	−1	0	0
	0	0	0
scid	**−1** (*n* = 1)	**+13** (*n* = 1)	**−6** (*n* = 1)
D			
Normal	**−5** (*n* = 1)	**+36** (*n* = 1)	**−69** (*n* = 1)
scid	**−16.5** (*n* = 2)	**+7** (*n* = 2)	**−94** (*n* = 2)
	−18	0	−123
	−15	+14	−65
E			
Normal	**−1** (*n* = 5)	**0** (*n* = 3)	**−126** (*n* = 3)
	−2	0	−2**
	0	0	−4**
	0		
	−2	0	−373**
scid	**−18** (*n* = 2)	**+35** (*n* = 2)	**−55** (*n* = 2)
	−18	+18	−2
	−17	+51	−107
Totals			
Normal	**−2.5** (*n* = 14)	**+18** (*n* = 11)	**−50** (*n* = 12)
scid	**−9.5** (*n* = 10)	**+16** (*n* = 10)	**−52** (*n* = 9)

[a] Individual recombination events are given in regular print and totals are given in bold print. *n*, number of events averaged for a given end. In those cases where the junction corresponds to positions of homology in each of the two DNA ends, the most 3′ deletion endpoints have been listed. *indicates 1-bp and ** indicates 2-bp homology at the junction. The smaller degree of loss at the *cat* gene end reflects the proximity of the *cat* gene which begins 47 bp from the free DNA end. The promoter is 83 bp from its designated end. The reason that losses at the promoter end can extend beyond the promoter is that upstream adventitious prokaryotic transcriptional promoters can direct transcripts into the *cat* gene.

Nucleotide addition occurred in almost half of the end joining events and, again, no marked differences were apparent between normal and scid fibroblasts. Repeating this analysis on a small collection of intramolecular end joinings in lymphocytes, no marked differences were noted between normal and scid (Table 6). Some of the nucleotide additions were over 100 bases in fibroblasts and lymphocytes (Tables 5 and 6). These additions are not GC rich, and terminal

TABLE 6. Summary of nucleotide loss and addition at recircularization junctions in lymphocytes

Phenotype	Nucleotide loss or addition[a]		
	cat gene end	Joint	Promoter end
Normal	**−4.2** (n = 5)	**+40** (n = 5)	**−100** (n = 6)
	−14	+130	−29
	0	+36	−89
	0	+31	−405
	−7	0	−58
			−17
	0	+3	−4
scid	**−6.3** (n = 4)	**+12** (n = 3)	**−116** (n = 3)
	−14	+36	−4
	0		
	−11	0	−331
	0	0	−14*

[a] Designations are as for Table 5. Individual events are given in regular print and totals are in bold print. As in Table 5, the smaller degree of loss at the *cat* gene end reflects the proximity of the *cat* gene which begins 47 bp from the free DNA end.

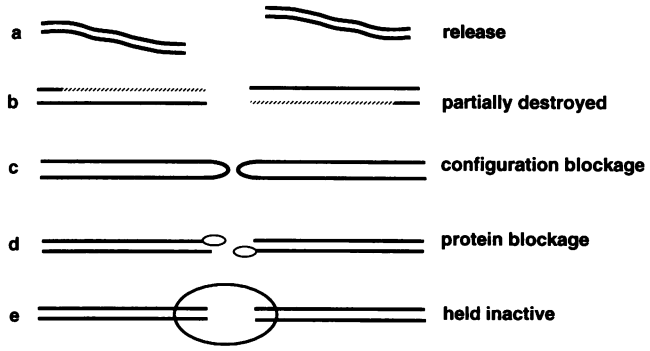

FIG. 6. Models for the end processing defect in scid. (a) DNA ends are released unjoined; (b) one strand of each DNA end is destroyed; (c) DNA ends have a hairpin that is not a substrate for DNA ligation; (d) covalent linkage of a protein to each DNA end blocks its resolution; (e) noncovalent binding of protein to each free end blocks its resolution.

transferase is not found outside of the hematopoietic system (11). Unlike most of the additions in V(D)J recombination, these nucleotide additions may arise from oligonucleotide capture (25).

We also examined the recircularization products for the possible usage of homology in alignment of the ends (Table 7). In contrast to simian fibroblasts in which 60% of end joining events utilize homology (25), only 7 of the 30 junctions analyzed from scid and normal murine fibroblasts and lymphocytes showed homology. The homology blocks were 2 bp in five cases and only 1 bp in two cases. The sites of homology were different in each of the seven cases, and the single-base homologies may be chance sites of joining without mechanistic significance. Therefore, utilization of homology in the alignment of DNA ends is less apparent in this study than in a similar analysis in simian fibroblasts.

DISCUSSION

Affected and unaffected end joining reactions in scid. The coding joint resolution of V(D)J recombination is reduced approximately 5,000-fold in scid lymphoid cells. Others have found that scid fibroblasts have a two- to threefold reduction in the rate of repair of double-strand breaks created by radiation and bleomycin (1, 6). Here, we have studied three other types of end joining in scid cells: integration of linear DNA, recircularization of linear DNA, and intermolecular end joining. We find that integration of linear DNA is reduced 11- to 75-fold in scid fibroblasts compared with that in normal cells. In contrast, recircularization and intermolecular end joining are neither quantitatively nor qualitatively affected.

Though the intra- and intermolecular end joining reactions are not affected by the *scid* mutation, they are still quite informative about the defect in scid. These results indicate that the defect in scid, at least as it relates to V(D)J recombination, is unlikely to result simply from an inability to retain the ends in the reaction intermediate (Fig. 6A). If this were the case, then at least 0.1 to 1% of these ends should be resolvable. But V(D)J recombination coding joint formation is reduced 5- to 50-fold below this. The end joining assays provide us with only lower limit estimates of the end joining efficiencies. Hence, this gap between the recircularization efficiency and the V(D)J coding joint frequency is likely to be even larger.

Given that premature release of the ends by itself appears to be an unlikely explanation of the defect in scid, it is possible that the DNA ends are not released but are in a state that does not allow resolution (Fig. 6B to E). Such impediments to resolution include double- or single-stranded exonucleolytic degradation of the two ends (Fig. 6B), nonreactive end configurations (Fig. 6C) (16), protein-linked ends (Fig. 6D), and protein-bound ends (Fig. 6E). On the basis of the ability of 70-bp homology blocks at coding ends in scid to undergo coding end resolution by homologous recombination, double-stranded exonucleolytic degradation is very unlikely (19). If single-stranded exonucleolytic degradation were occurring, then one would expect short homology blocks to rescue scid coding ends. We have tested for exposure of single-stranded character at the coding ends by providing short regions of homology ranging from 3 to 24 nucleotides in length and recessed various distances from each coding end (2a). We have not seen any indication that these short regions of homology are utilized in scid (Fig. 6B).

We also consider it unlikely that the *scid* defect in V(D)J recombination is due to the holding of the DNA ends in an inert configuration (Fig. 6E). If this were the case, it would be difficult to reconcile how recombination between 70-bp homology blocks at coding ends could occur, and it would be difficult to reconcile hybrid joint formation in scid lymphoid cells (Results) (18).

Configurational (Fig. 6C) and protein-linkage (Fig. 6D) blocks are the remaining categories of end resolution failure possible in scid. An uncleavable protein-linkage in scid is difficult to reconcile with the extensive nucleotide loss at hybrid joints in scid (18). Moreover, nucleotide additions at hybrid joints are GC rich, which is consistent with the action by terminal transferase acting at a free end (17 [and references cited therein], 18).

We favor a hairpin configurational blockage in scid because it would explain the inverted repeats found at the rare coding joints that are recovered from scid lymphoid cells (26, 27). Inverted repeats have been described at the coding joints in normal murine lymphoid T-cell receptor and immunoglobulin gene rearrangements (12, 21). On the basis of the inverted repeats found in normal and rare scid coding joints, we have proposed a model for their formation that involves hairpin formation as an intermediate in V(D)J recombination

TABLE 7. Homology usage and nucleotide addition at recircularization junctions in normal and scid fibroblasts and lymphocytes

Cell type and phenotype	Homology usage[a]	Nucleotide addition[b]
Fibroblast		
Normal	5/12	4/12
scid	1/10	5/10
Total	6/22	9/22
Lymphocyte		
Normal	0/5	4/5
scid	1/3	1/3
Total	1/8	5/8

[a] Homology usage was demonstrated in 5 of 17 normal and 2 of 13 scid cell products.

[b] Nucleotide addition was demonstrated in 8 of 17 normal and 6 of 13 scid cell products.

(16). The failure in scid to efficiently open up such hairpin coding ends would explain their inefficiency in joining together and yet be consistent with a longevity that is sufficient to allow them to undergo homologous recombination (18). Most of these chromosomal breaks may lead to cell death. Rare ones may resolve because of random breaks recessed hundreds or thousands of base pairs from each hairpin end, explaining the large deletions at the antigen receptor loci in transformed lymphoid cells from scid mice (5, 10, 20, 22, 23, 27, 28).

How do we relate the deficits in V(D)J recombination, ionizing radiation and bleomycin repair, and DNA integration? The mechanism of generation of DNA ends is uncertain for the genomic integration sites in exogenous DNA integration and for ionizing radiation and bleomycin. Free radical mechanisms of DNA damage are thought to be involved for the latter two and may be involved at sites of spontaneous genomic breakage (11). Whether or not repair of free radical-generated DNA ends involves a hairpin intermediate is a matter of speculation, but it is a hypothesis that we are currently testing.

It would be desirable to compare a larger number of scid and normal fibroblast lines to eliminate any possibility that line-to-line variability in the DNA integration analysis might be artifactually creating the differences that we observe. In this regard, it is noteworthy that DNA integration (after calcium phosphate transfection and with a G418 selection beginning 48 h afterward) in a C.B-17 *scid* fibroblast line distinct from ours was also 10- to 100-fold lower than a normal fibroblast line (1).

It is interesting that a comparison of retroviral integration efficiencies between normal (NF-22) and *scid* (SF-7) fibroblasts showed them to be within a factor of 2 of one another (3a). The retroviral integration reaction involves the generation of a genomic double-strand break having 5′ overhangs. However, the generation of the double-strand break and the joining of it to the ends of the retrotransposon appear to be a concerted reaction. Hence, there is little or no opportunity for exposure of these genomic ends to any proteins outside of the integrase complex (11).

How leaky is the scid phenotype for normal coding joint formation? Early in the characterization of scid mice, it became clear that 10 to 30% of the mutant mice gave rise to a few clones of B cells that could each produce an immunoglobulin heavy and light chain (26). This was termed the leaky phenotype because it appeared that some penetration through the mutation was occurring. A number of laboratories have provided examples of junctions from scid cells that satisfy the normal criteria for coding joints (4, 26). It has not been possible to calculate the frequency of these events, and this has made it difficult to determine whether some residual level of coding joint formation could be the basis for the leaky phenotype. This issue is made more complex by the fact that normal DJ, then V to DJ, and finally, VJ_L joins must be completed by a single mutant cell in order to produce an antibody. The fact that any residual level of coding joint formation appears to be 5,000-fold reduced raises the question of whether this low level could account for the leaky phenotype of scid. Two successful joins at the heavy-chain locus would occur in one of $(5{,}000)^2$ or 2.5×10^7 cells. Estimates are that 10^7 new pre-B cells appear in the murine bone marrow per day. Hence, a pre-B cell expressing cytoplasmic mu chain might be expected to appear every 1 to 3 days. Whether these cells propagate after heavy-chain rearrangement but before light-chain rearrangement affects the likelihood that the rare formation of a productive heavy chain in these cells will be followed by a productive light-chain rearrangement. Hence, the marked reduction in coding joint formation may still allow for very rare cells that are fully assembled at their antigen receptor genes as suggested previously (5), and the frequency of these cells may be influenced by the mitotic rate of pre-B cells at various stages along the differentiation pathway.

Studies about the types of reactions affected and the magnitude of the affects in scid are important as we try to understand general and site-directed recombination in higher eukaryotes and as various strategies are carried out to isolate the *scid* gene.

ACKNOWLEDGMENTS

We thank Mel Bosma for comments on the manuscript. We thank Greg Duda for excellent technical assistance.

This work was supported in part by NIH grant GM43236 to M.L. M.L. is a Lucille P. Markey Scholar, and this work was supported in part by a grant from the Lucille P. Markey Charitable Trust.

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