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Strand Breaks without DNA Rearrangement in V(D)J Recombination

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Somatic gene rearrangement of immunoglobulin and T-cell receptor genes [V(D)J recombination] is mediated by pairs of specific DNA sequence motifs termed signal sequences. In experiments described here, retroviral vectors containing V(D)J rearrangement cassettes in which the signal sequences had been altered were introduced into wild-type and *scid* (severe combined immune deficiency) pre-B cells and used to define intermediates in the V(D)J recombination pathway. The *scid* mutation has previously been shown to deleteriously affect the V(D)J recombination process. Cassettes containing a point mutation in one of the two signal sequences inhibited rearrangement in wild-type cells. In contrast, *scid* cells continued to rearrange these cassettes with the characteristic *scid* deletional phenotype. Using these mutated templates, we identified junctional modifications at the wild-type signal sequences that had arisen from strand breaks which were not associated with overall V(D)J rearrangements. Neither cell type was able to rearrange constructs which contained only a single, nonmutated, signal sequence. In addition, *scid* and wild-type cell lines harboring cassettes with mutations in both signal sequences did not undergo rearrangement, suggesting that at least one functional signal sequence was required for all types of V(D)J recombination events. Analysis of these signal sequence mutations has provided insights into intermediates in the V(D)J rearrangement pathway in wild-type and *scid* pre-B cells.

Functional immunoglobulin and T-cell receptor genes are assembled from separate gene elements via somatic gene rearrangement [V(D)J recombination] during lymphoid differentiation (5, 21). The gene elements targeted for rearrangement are flanked by conserved signal sequences which mediate the rearrangement event. These signal sequences consist of highly conserved heptamer and nonamer elements separated by a spacer of either 12 or 23 bp (1). The subsequent rearrangement event generates two products: a signal junction and a coding junction. The signal junction consists of the precise head-to-head joining of the signal sequences, whereas the coding junction formed from the coding strands is marked by the variable loss and/or addition of nucleotides.

Early models of V(D)J recombination proposed that rearrangement is initiated by the recognition and DNA-DNA pairing of the signal sequences (8, 28), followed by the introduction of double-stranded breaks (DSBs) at the heptamer-coding element junctures (2). These models of V(D)J recombination have been modified to account for the existence of nonrandom nucleotides at coding joints (P nucleotides [19]) as well as two alternative products of the V(D)J joining process: hybrid junctions, in which a signal end is incorrectly joined to a coding joint end instead of another signal end (22, 25), and open/shut junctions, in which initiation of recombination, junctional diversification, and resealing of the heptamer/coding element juncture occur without gene rearrangement (22). In addition, extensive mutational analysis of the signal sequence domains demonstrated that alteration of any of the first 3 nucleotides (nt) of the heptamer sequence (CACAGTG) reduced recombination frequency by 2 orders of magnitude, suggesting that these nucleotides are absolutely critical for mediating efficient recombination (1, 15). Compensatory mutations that restored signal heptamer complementarity, however, did not restore the ability to undergo V(D)J rearrangement (15). In this regard, lymphoid gene rearrangement appears to be fundamentally different from other site-specific systems such as Flp (27, 30), Hin (16), and lambda (for a recent review, see reference 20) in which compensatory mutations in the respective recombination signals are tolerated quite well and are thought to facilitate DNA-DNA pairing. Thus, rather than promoting DNA-DNA pairing, the lymphoid signal sequences might represent specific binding sites for recombinational proteins (15).

Mice homozygous for the *scid* (severe combined immune deficiency) mutation lack detectable mature B and T lymphocytes as the result of an apparent V(D)J recombination defect (7, 29). Analysis of Abelson murine leukemia virus-transformed *scid* pre-B cells (13, 17, 24, 29), cultured *scid* bone marrow cells (26), and spontaneous *scid* T-cell lymphomas (29) showed that large deletions accompanied rearrangement events. These deletions removed all or most of the coding sequences (6, 9, 13, 14, 23, 24) and resulted in lymphoid cells incapable of synthesizing immunoglobulin heavy-chain, immunoglobulin light-chain, or T-cell receptor proteins. Recently, it has been shown that the *scid* gene product is also expressed in nonlymphoid tissues (11, 12) and is involved in the repair of DNA DSBs (12). Thus, we have proposed that the *scid* gene product is one of the proteins responsible for repairing the DSBs associated with coding joint formation during V(D)J recombination (12).

We report here studies of continuing gene rearrangement events in *scid* and wild-type pre-B cells that delineate the requirement for certain intermediates in V(D)J recombination. A series of retroviruses containing V(D)J rearrangement cassettes carrying mutated signal sequences were introduced into *scid* and wild-type pre-B cell lines. Dele-

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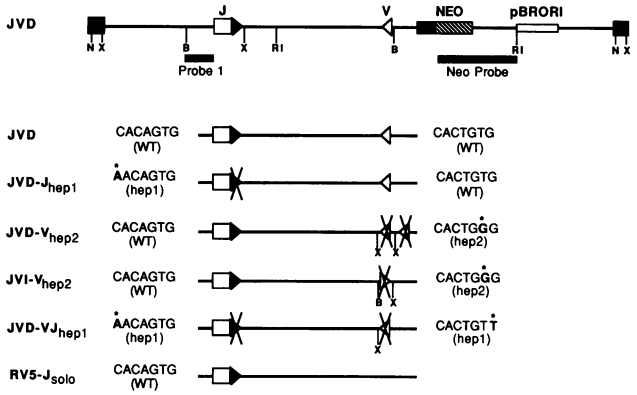


FIG. 1. Retroviral vectors containing wild-type and mutated signal sequences used to assay V(D)J recombination. The parent retrovirus JVD is shown on the top line. Symbols: shaded square, 5′ long terminal repeat; open square, J coding sequences; black triangle, J signal sequences; open triangle, V signal sequence; filled rectangle, simian virus 40 promoter/enhancer; hatched rectangle, *neo* gene; open rectangle, pBR322 origin of replication; shaded square, 3′ long terminal repeat. Underneath are shown relevant restriction enzyme sites (N, *Nhe*I; X, *Xba*I; B, *Bam*HI; RI, *Eco*RI) and the two regions which were used as Southern probes: probe 1 and the *neo* probe (solid rectangle). Below, in abbreviated form, are shown the signal sequence derivatives. The wild-type (WT) JVD heptamer sequence is shown; the starred, bold nucleotides correspond to the mutations (schematically shown by a large X through the affected heptamer).

tional activity of the *scid* defect could be detected at all DSBs introduced by the V(D)J recombination system, including DSBs that could not lead to rearrangement due to a heptamer mutation in one of the signal sequences. Heptamer mutations in both signal sequences totally abrogated the *scid* deletional phenotype and also reduced wild-type open/shut events to undetectable levels. These experiments pinpoint intermediates in the V(D)J rearrangement pathway.

MATERIALS AND METHODS

Construction of retroviral recombination cassettes. Retroviruses containing V(D)J recombination templates were prepared in plasmid pDOL, containing a retroviral provirus and the G418 resistance gene, *neo*, expressed from a flanking simian virus 40 promoter/enhancer (18). Two plasmids were constructed, pLT2-7 (JVD) and pLTH-15 (JVD-J$_{hep1}$), containing a single mouse J$_{\kappa 1}$ coding region and flanking signal sequence and a single V$_{\kappa 21}$ signal sequence on a 3.5-kb *Bam*HI fragment, inserted at the *Bam*HI site of pDOL. The signal sequences were oriented for deletional V(D)J rearrangement. pLTH-15 contained a mutated J signal at heptamer position 1 (JVD-J$_{hep1}$; Fig. 1).

Point mutations were introduced into the V heptamer-nonamer of JVD and JVD-J$_{hep1}$. JVD and JVD-J$_{hep1}$ were linearized by partial digestion with *Bam*HI, one restriction site of which resides only 3 nt from the V signal sequence. Linear DNA molecules were purified from agarose gels and then treated with *Bal*31 nuclease under limiting conditions to remove the region of the wild-type V signal sequence (<200 nt). Digested DNA was then ligated to *Sal*I linkers, redigested with *Sal*I, and circularized with T4 DNA ligase. Following transformation into *Escherichia coli* and analysis of miniprep plasmid DNA, clones (termed 7-12 and 15-13) were chosen where the *Bal*31 deletion of the V signal sequence had been achieved. Concurrently, the heptamer point mutations were generated by gap duplex mutagenesis in plasmid pYJV5, which contained a V$_{\kappa}$ wild-type heptamer-nonamer element on a 300-nt *Sal*I fragment. Using 16-mer oligonucleotides synthesized with mutations at heptamer residue 1 or 2, gap repair incorporated the mutated oligonucleotides and formed double-stranded plasmid circles in vitro as described previously (10). Following transformation into *E. coli*, colonies were screened for the mutated oligonucleotide by standard colony hybridization techniques. The signal heptamer DNA sequence of positive recombinants was determined. Then *Sal*I fragments containing the mutated heptamer sequences were introduced into the unique *Sal*I sites of plasmids 7-12 and 15-13 to create plasmids containing combinations of mutated and wild-type heptamers as depicted in Fig. 1. The orientation of the *Sal*I fragment determined whether the resulting cassettes underwent deletional (JVD [J-to-V deletional]) or inversional (JVI [J-to-V inversional]) V(D)J rearrangement.

RV5-J$_{solo}$ was constructed from the retroviral template vector pLNL6 (4). pLNL6 was cleaved at the unique *Xho*I site in the polylinker, and a 450-bp fragment containing the J$_{\kappa 1}$ gene element/signal sequence and a V$_{\kappa}$ heptamer-nonamer sequence was inserted. This derivative was then digested with *Hin*dIII to remove the V$_{\kappa}$ signal sequence region and resealed. Thus, the plasmid contains a proviral insert with a single J$_{\kappa 1}$ gene element/signal sequence and the *neo* gene for G418 resistance selection.

Recombinant virus infections. The recombinant retroviruses were produced from the proviral constructs described above. Plasmids were introduced by standard $CaPO_4$-mediated transfection of 10^6 Ψ2 or PA317 fibroblast retroviral packaging cells, and the virus was harvested 18 h after transfection. These virus stocks were directly added to 100-mm tissue culture dishes containing 10^6 Ψ2 or PA317 cells for 12 h, and stable virus-producing cell lines were isolated by G418 resistance (0.2 to 0.4 mg/ml [13]).

Abelson murine leukemia virus-transformed pre-B cells derived from either wild-type (38B9 and 300-19 [2]), homozygous *scid* [8D [13]), or heterozygous *scid* (SN-1 [23]) mice were infected either by direct infection or by cocultivation with fibroblast retrovirus producer cell lines (13). In addition, NIH 3T3 cells were infected with virus to determine the incidence of deletion mutants in the stocks. Clonality of the cells was judged on the basis of viral insertion sites as determined by Southern blot analysis.

Southern blots. Procedures for preparation of genomic DNA, labeling of probes, and Southern blot analysis have been described (13).

PCR analysis of open/shut rearrangements. Genomic DNA was purified from individual clones, and then the polymerase chain reaction (PCR) was conducted by using 1 μg of DNA in a 25-μl reaction mix containing 50 mM KCl, 10 mM Tris (pH 8.3), 1.0 mM $MgCl_2$, 0.1% gelatin, 200 mM deoxynucleoside triphosphates, and 25 pmol of each oligonucleotide (primer 72 and primer 1252). PCR was carried out for 40 cycles at 94°C for 1 min, 50°C for 1.5 min, and 72°C for 2 min. PCR products were purified from agarose gels, cloned into pUC19, and screened by colony hybridization as described previously (14) except that the filters were subjected to two rounds of screening, first with oligo 1855 and subsequently with oligo 2519. Hybridization was at room temperature in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1× Denhardt's solution for 16 h. Wash conditions (6× SSC, 0.1% sodium dodecyl sulfate) for colony hybridizations were at room temperature (once), 37°C (once), and

45°C (twice), each for 10 min. Colonies which hybridized to oligo 1855 but not to oligo 2519 (see Fig. 4) were picked, and miniprep plasmid DNA was analyzed by restriction digests and then DNA sequencing as described previously (14). Alternatively, PCR reactions were performed with genomic DNA from JVD-J_{hep1} and JVD-VJ_{hep1} templates in the same manner. Oligo 124, which was complementary to the J region junction with a hep1 mutant signal sequence, was substituted for oligo 2519 in the screen for open/shut events at the mutated signal sequence of JVD-J_{hep1} (see Results). The oligonucleotide primers synthesized were as follows: primer 72, TGGACAGCATGGCCTGCAAC; primer 1252, GGAA TTCCTCGGCGAGAAGCAGGCCATT; oligo 1855, GCCG GAAGCGAGAAGAATCA; oligo 2519, CCACTGTGGTG GACGT; and oligo 124, CCACTGTTGTGGACGT.

RESULTS

Retroviruses containing V(D)J rearrangement cassettes that carried altered signal sequences were introduced into wild-type and *scid* pre-B cells to examine the mechanism of V(D)J gene rearrangement. The parent vector, JVD, contained wild-type V and J signal sequences oriented such that DNA rearrangement would form coding junctions on the template and delete the intervening DNA including the signal junction (Fig. 1). When introduced into wild-type pre-B cells, JVD underwent the expected deletional V(D)J rearrangement event (data not shown). JVD introduced into *scid* pre-B cells also underwent deletional rearrangements, but these were accompanied by additional aberrant deletions of the template, consistent with the previously characterized phenotype of the *scid* mutation (data not shown; 6, 9, 13, 14, 17, 23, 24, 26, 29).

Single-point mutations in signal sequence heptamers affect V(D)J rearrangements in *scid* and wild-type pre-B cells differently. Heptamer positions 1 through 3 of the signal sequence are highly conserved, and mutations at these positions of either signal sequence essentially abolish rearrangement (1, 15). Heptamer point mutations in either position 1 or 2 of JVD were constructed (see Materials and Methods and Fig. 1). To investigate what effect a point mutation in a heptamer element would have on V(D)J rearrangement, wild-type, *scid* heterozygous, and *scid* homozygous pre-B cells were infected with JVD-J_{hep1}, JVD-V_{hep2}, and JVI-V_{hep2} viruses (Fig. 1). Cells were positively selected for G418 resistance conferred by the constitutive expression of the *neo* gene in the virus. Subsequently, the V(D)J rearrangement of JVD in these cell clones was assessed by Southern blotting with probe 1 following digestion of genomic DNA with *Bam*HI (Fig. 1). The rearrangement cassettes of JVD-J_{hep1} and JVD-V_{hep2} were contained on a single 3.5-kb *Bam*HI fragment. For JVI-V_{hep2}, the V signal sequence lies only 3 nt away from the *Bam*HI restriction site, and therefore any deletion larger than 3 nt would be detected by using *Bam*HI and probe 1 in Southern blots. In wild-type cells, as has been previously observed (1, 15), the introduction of a point mutation into the heptamer at residue 1 blocked normal DNA rearrangement, because only the unrearranged 3.5-kb *Bam*HI fragment was observed (Fig. 2A-C, Wild-type). In fact, any of the three single heptamer mutations inhibited wild-type rearrangement events, regardless of which signal sequence was mutated and regardless of whether the signal sequences were in deletional or inversional orientation (Table 1).

Surprisingly, a significant percentage of the *scid* clones isolated from single heptamer mutation virus infections

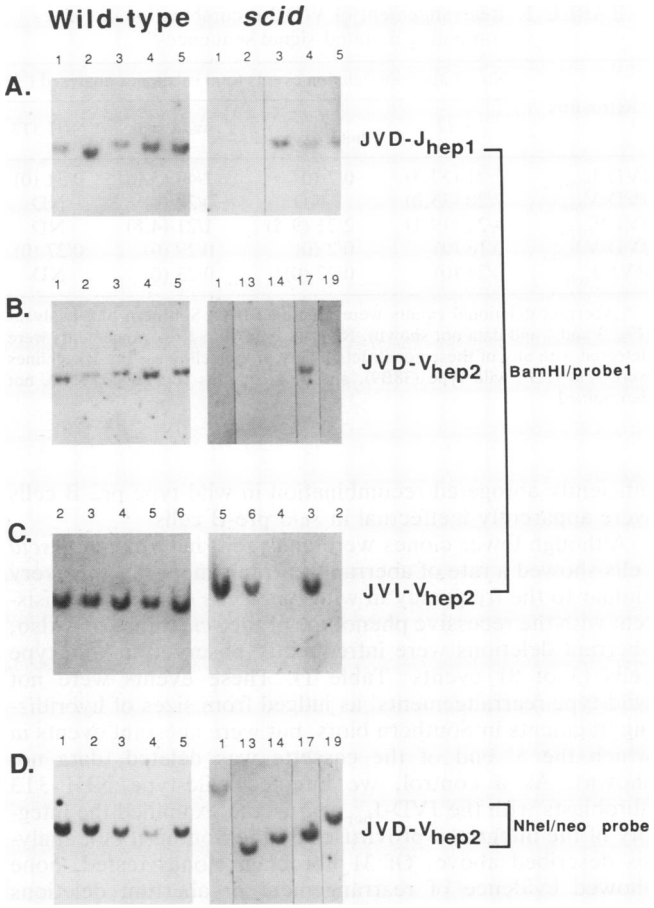


FIG. 2. Evidence that mutation in one of the signal sequences abolishes V-to-J rearrangement in wild-type cells but does not block the generation of deletions in *scid* pre-B cells. Wild-type (38B9) and *scid* (8D) pre-B cells were infected with JVD-J_{hep1} (A), JVD-V_{hep2} (B and D), and JVI-V_{hep2} (C) (Fig. 1), and individual clones were analyzed by Southern blotting using either *Bam*HI and probe 1 (A to C) or *Nhe*I and the *neo* probe (D). Numbers above the gel lanes correspond to cell clone numbers. The *scid* JVD-V_{hep2} clone 17 is included in panels B and D as a positive control clone which did not rearrange.

contained no probe 1-hybridizing DNA, as shown with the same Southern blot analysis (Fig. 2A to C, *scid*; Table 1). These results were indicative of aberrant deletional rearrangements, similar to what we observed with the wild-type rearrangement cassette (JVD). To verify this, genomic DNA from JVD-V_{hep2} cell clones was digested with *Nhe*I (which cleaves only in the long terminal repeats; Fig. 1) and probed with the *neo* probe. All of the cassettes analyzed from wild-type cells contained only the unrearranged 6.2-kb fragment (Fig. 2D, Wild-type), consistent with the Southern analyses described above. *scid* clones, however, contained only part of the JVD-V_{hep2} rearrangement cassette and in addition either contained large internal deletions or deletions into chromosomal DNA that removed the entire 5′ end of JVD-V_{hep2} (Fig. 2D, *scid*; data not shown). For the three single point mutant viruses tested, a total of 18 of 62 clones (29.0%) in *scid* pre-B cells demonstrated aberrant rearrangements, which was eightfold higher than the level observed in wild-type cells (Table 1). Thus, heptamer mutations that

TABLE 1. Rearrangement of V(D)J recombination cassettes containing mutated signal sequences[a]

Retrovirus	No. of aberrant deletion events/total subclones analyzed (%)			
	scid	*scid* heterozygous	Wild type	NIH 3T3
JVD-J_{hep1}	7/21 (33.3)	0/7 (0)	2/40 (5.0)	0/31 (0)
JVD-V_{hep2}	7/20 (35.0)	ND	0/20 (0)	ND
JVI-V_{hep2}	4/21 (19.1)	2/21 (9.5)	1/21 (4.8)	ND
JVD-VJ_{hep1}	0/16 (0)	0/2 (0)	0/29 (0)	0/27 (0)
RV5-J_{solo}	0/24 (0)	0/22 (0)	0/23 (0)	ND

[a] Aberrant deletional events were identified from Southern blot analyses (Fig. 3 and 4 and data not shown). No wild-type V-to-J rearrangements were detected with any of these constructs in any of the cell lines. Pre-B cell lines were *scid* (8D), wild type (38B9), and heterozygous *scid* (SN-1). ND, not determined.

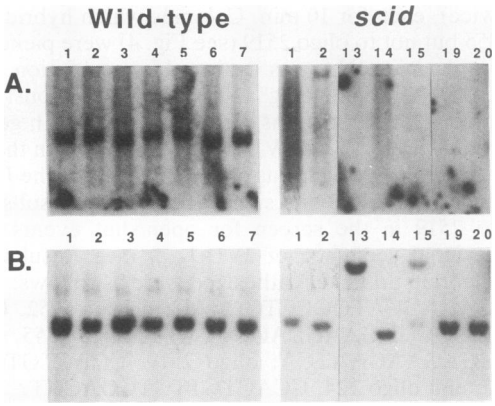


FIG. 3. Evidence that deletions of JVD-V_{hep2} in *scid* pre-B cells always include the nonmutated signal sequence. Genomic DNA from individual clones was treated with *Xba*I and then subjected to Southern analysis using probe 1 (A). Alternatively, genomic DNA was treated with *Eco*RI and then subjected to Southern analysis with the *neo* probe (B). See Fig. 1 for positions of restriction sites and probes. Numbers above the lanes refer to cell clone numbers.

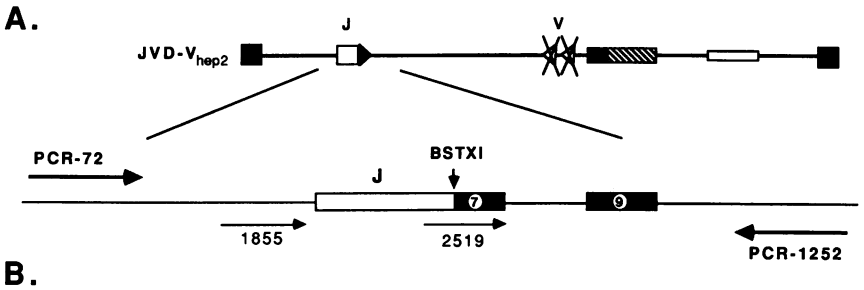
efficiently abrogated recombination in wild-type pre-B cells were apparently ineffectual in *scid* pre-B cells.

Although fewer clones were analyzed, heterozygous *scid* cells showed a rate of aberrant rearrangement that was very similar to the frequency in wild-type cells (Table 1), consistent with the recessive phenotype of the *scid* mutation. Also, aberrant deletions were infrequently observed in wild-type cells (3 of 81 events; Table 1). These events were not wild-type rearrangements, as judged from sizes of hybridizing fragments in Southern blots, but were aberrant events in which the 5′ end of the cassette was deleted (data not shown). As a control, we infected wild-type NIH 3T3 fibroblasts with the JVD-J_{hep1} virus and examined the integrity of the integrated proviruses by the Southern blot analysis described above. Of 31 fibroblast clones tested, none showed evidence of rearrangement or aberrant deletions (Table 1). Thus, the altered rearrangements of these cassettes in wild-type pre-B cells did not result from deletion derivatives preexisting in the virus stocks and were not generated during the process of infection and integration.

Point mutations in each of a pair of heptamer-nonamer recognition elements were previously observed to diminish the level of V(D)J rearrangement in wild-type pre-B cells (15). Wild-type, *scid*, and *scid* heterozygous pre-B cells were infected with JVD-VJ_{hep1}, a retroviral cassette in which both signal sequences were mutated (Fig. 1). Individual G418-resistant clones were digested with *Bam*HI and hybridized with probe 1 by Southern blots as described above. No aberrant *Bam*HI fragments or large deletions were detectable by Southern blotting in any of the three cell lines (Table 1).

Aberrant deletions in *scid* pre-B cells originate from the wild-type signal sequence. The unexpected ability of *scid* pre-B cells to rearrange single heptamer mutants may have arisen by initiation of rearrangement from the wild-type signal sequence present in the cassette. To test this hypothesis, genomic DNAs from JVD-V_{hep2} clones were digested with either *Xba*I or *Eco*RI, and the resulting Southern blots were hybridized with probe 1 or the *neo* probe, respectively. The 1.3-kb *Xba*I fragment hybridizing with probe 1 scores for the retention of an unrearranged J signal sequence, and the 3.1-kb *Eco*RI fragment hybridizing with the *neo* probe scores for the presence of the V signal sequence (Fig. 1). Wild-type cells showed no rearrangement of either of the two signal sequence regions in this more specific Southern blot analysis (Fig. 3, Wild-type). In contrast, six of seven *scid* clones that were scored as aberrant in the previous Southern analyses (see above) did not contain *Xba*I fragments hybridizing to probe 1 (Fig. 3A, *scid*; data not shown). In all of these cases the wild-type J signal sequence had been deleted. Five of the seven *scid* clones, however, did retain the unrearranged 3.1-kb *Eco*RI fragment hybridizing to the *neo* probe, the region containing the mutated signal sequence (Fig. 3B). One *scid* clone exhibited a probe 1-hybridizing fragment of greater than 12.9 kb, while the *Eco*RI/*neo* pattern showed no rearrangement (Fig. 3, clone 2), suggesting that this clone had recombined with chromosomal DNA sequences. These results suggested that the *scid* deletions observed on the singly mutated rearrangement cassettes were initiated at the wild-type signal sequence followed by aberrant deletions. In some cases, these deletions appeared to extend into the region of the mutated signal sequence as well (Fig. 3B, *scid*, clones 13 and 15). In support of this view, in previous experiments using another retroviral rearrangement template (DGR) in *scid* pre-B cells, we observed a deletion that removed only one of the three wild-type signal sequences in this cassette (DGR 4-6-15 [14]). This partial deletion in DGR could not have been part of the normal rearrangement mechanism but was also consistent with aberrant activity at one signal sequence only.

Cleavage without rearrangement is observed at the wild-type signal sequence in singly mutated cassettes. Lewis et al. (22) have described open/shut recombination in which cleavage occurred at a single signal sequence, followed by junctional diversification (addition and deletion of nucleotides), and then rejoining of the same coding and signal ends. Consequently, there was no overall V-to-J rearrangement, even though both signal sequences were wild type and capable of standard V-to-J rearrangement. Since the *scid* deletions always removed the wild-type signal sequence (Fig. 3), we hypothesized that these events were caused by an open/shut reaction with the deletional *scid* phenotype superimposed upon it. Because the experiments of Lewis et al. (22) were performed with constructs containing two wild-type signal sequences, it was imperative to show that open/shut events could occur on cassettes in which only one of the signal sequences was functional. Therefore, a PCR assay was developed to isolate and analyze open/shut events at a wild-type heptamer/J coding element junction in a retroviral cassette containing a mutated V signal sequence



B.

J CODING SEQUENCE	N	7	23-SPACER	9	J	N	7
GCTTGGTGCCTCCACCGAACGTCCAC		CACAGTG	GTAGTACTCCACTGTCTGGCTGT	ACAAAAACC	0	0	0
GCTTGGTGCCTCCACCGAACGTCCA		CACAGTG	GTAGTACTCCACTGTCTGGCTGT	ACAAAAACC	-1	0	0*
GCTTGGTGCCTCCACCGAACGTCCAC	GT	CACAGTG	GTAGTACTCCACTGTCTGGCTGT	ACAAAAACC	0	+2	0
GCTTGGTGCCTCCACCGAACGTC		CACAGTG	GTAGTACTCCACTGTCTGGCTGT	ACAAAAACC	-3	0	0*
GCTTGGTGCCTCCACCGAACGTCCAC	A	ACAGTG	GTAGTACTCCACTGTCTGGCTGT	ACAAAAACC	0	+1	-1
GCTTGGTGCCTCCACCGAACGTCCAC	G	CACAGTG	GTAGTACTCCACTGTCTGGCTGT	ACAAAAACC	0	+1	0
GCTTGGTGCCTCCACCGAACGTCC	CCCT	ACAGTG	GTAGTACTCCACTGTCTGGCTGT	ACAAAAACC	-2	+4	-1

C.

GCTTGGTGCCTCCACCGAACGT	CC	CACAGTG	GTAGTACTCCACTGTCTGGCTGT	ACAAAAACC	-4	+2	0
GCTTGGTGCCTCCACCGAACGTCC		CACAGTG	GTAGTACTCCACTGTCTGGCTGT	ACAAAAACC	-2	0	0
GCTTGGTGCCTCCACCG	GC		CTGTCTGGCTGT	ACAAAAACC	-9	+2	-18
GCTTGGTGCCTCCACCGAACGT	AGTCCA		CCACTGTCTGGCTGT	ACAAAAACC	-4	+6	-15

FIG. 4. Detection of junctional diversification at wild-type signal sequences in rearrangement-blocked cassettes in wild-type and *scid* pre-B cells. The top line shows the JVD-V_{hep2} vector. The panel underneath shows the region surrounding the J element and its signal sequence in JVD-V_{hep2}. The two PCR primers (bold arrows) and the two oligonucleotides (1855 and 2519; thin arrows) used to screen the cloned PCR products are shown below. The position of the *Bst*XI site, cleavage of which facilitated removal of background unrearranged templates, is shown as a small arrow at the coding/signal junction. Below are shown the open/shut events recovered from wild-type (B) and *scid* (C) pre-B cells. The sequence of the J coding element and its signal sequence for unrearranged JVD-V_{hep2} are shown on the top line of panel B. The highly conserved heptamer and nonamer elements of the signal sequence are underlined. The type and amount of deletion or insertion (N region) is shown in the underlying sequences and in tabular form on the right. It should be noted that because of the redundancy between the 3′ end of the J coding sequence and the 5′ end of the signal sequence (i.e., CAC), the deletions in several of the constructs cannot be unambiguously assigned to a particular strand (*).

(JVD-V_{hep2}; see Materials and Methods). Genomic DNA from JVD-V_{hep2} cell clones was subjected to the PCR reaction using two primers that flank the J region and hybridize to opposite strands (primers 72 and 1252; Fig. 4A). To eliminate background, genomic DNA was first treated with the restriction enzyme *Bst*XI, the recognition site of which spans the J coding end/signal sequence junction of JVD-V_{hep2}. Unrearranged, wild-type junctions should be cleaved by *Bst*XI, making the resultant DNA fragments refractory to amplification. Open/shut reactions that destroy the *Bst*XI restriction site will allow PCR amplification of this region. PCR products were cloned into pUC19 and sequentially screened with oligo 1855, which hybridized to sequences internal to the amplified region and then to oligo 2519, which hybridized to the J coding end/signal sequence junction (Fig. 4A). Under our hybridization and wash conditions, J elements that had undergone even single base changes in the junction should not have hybridized to oligo 2519. Therefore, we sequenced those clones that hybridized to oligo 1855 but not to oligo 2519.

To identify open/shut events, we analyzed JVD-V_{hep2} clones that apparently had not undergone either rearrangement or aberrant deletion as determined by Southern blot analysis (Fig. 2 and 3). Open/shut events were readily detected in the JVD-V_{hep2} clones from wild-type pre-B cells (Fig. 4B). Small insertions (1 to 4 nt) and small deletions (1 to 3 nt) were observed precisely at the heptamer/J coding element junction even though one heptamer of the pair was mutated. The extent of junctional diversity in these reactions is reminiscent of the variability in rearranged coding junctions produced from V(D)J rearrangements in wild-type cells. Importantly, open/shut events were also detected from *scid* pre-B cells (Fig. 4C). Of the four events characterized, two resembled wild-type cleavage events repaired with small insertions and deletions (2 to 4 nt). The other two *scid* events had much larger deletions (up to 18 nt), consistent with the phenotype of the *scid* mutation. We also screened for open/shut events at the mutated signal sequence of JVD-J_{hep1}, using the same PCR approach but substituting a oligo 124, which hybridizes to the heptamer point mutation sequence at the junction, in place of oligo 2519 in the screening procedure (Materials and Methods). We were unable to detect any open/shut events at mutated signal heptamers. In summary, these results showed that the open/shut reaction could occur at a wild-type signal sequence even when the other signal sequence was altered by mutation. Second, the *scid* phenotype could be observed at single DSBs as part of the open/shut reaction.

As a control, one of the doubly mutated JVD-VJ_{hep1} cassettes was cloned from a *scid* cell line, and the DNA

sequences of the regions surrounding the signal sequences were obtained to verify that the rearrangement cassette was unaltered (data not shown). We were unable to detect open/shut events from genomic DNA of multiple JVD-VJ_{hep1} clones by using the PCR assay described above. If open/shut rearrangements occurred with JVD-VJ_{hep1}, they must have occurred at a frequency significantly lower than with JVD-V_{hep2}. In addition, attempts to detect coding junction formation by using a PCR assay were unsuccessful with all three cell types (31). Finally, retroviral cassettes oriented for either deletional or inversional rearrangement that contained point mutations at the first position of the J heptamer and the second position of the V heptamer were inert at the level of Southern analysis when introduced into wild-type pre-B cells (data not shown). Thus, *scid* pre-B cells required the presence of at least one functional signal sequence in order to exert its deletional phenotype and wild-type pre-B cells require at least one functional signal sequence to undergo any V(D)J recombination reaction.

One wild-type signal sequence is not sufficient for recombinational events. We introduced a construct containing one wild-type signal sequence only (RV5-J_{solo}; Fig. 1) into wild-type, *scid*, and *scid* heterozygous pre-B cell lines. Given the results of the foregoing experiments, we anticipated that RV5-J_{solo} would undergo open/shut events in wild-type cells and deletional events in *scid* cells. Instead, no rearrangement events or large deletions were observed in 69 RV5-J_{solo} clones, as determined by Southern blots (Table 1). As with JVD-VJ_{hep1}, we were also unable to detect any open/shut events from RV5-J_{solo} templates even though multiple subclones were assayed. These data indicated that open/shut events at a functional signal sequence may require the presence of another signal sequence, even one containing heptamer point mutations.

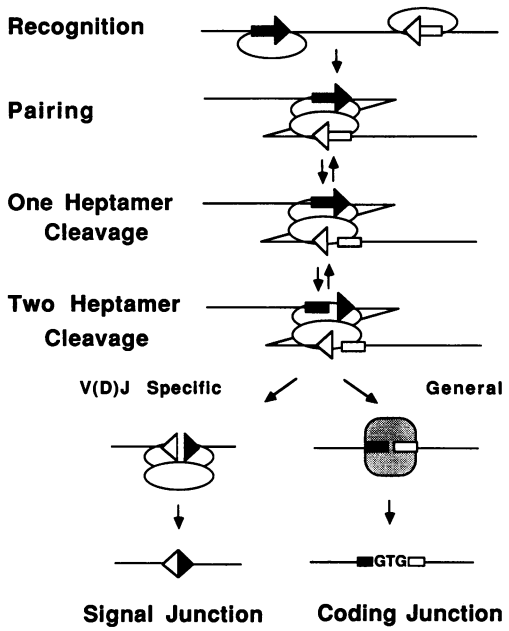


FIG. 5. Model for V(D)J recombination. The rectangles represent gene coding elements, and the triangles represent their corresponding signal sequences. The egg-shaped circles represent the V(D)J recombinase complex that may consist of multiple proteins. The stippled rectangle with rounded corners represents a general DNA repair complex that is postulated to be responsible for the processing of the coding junction. The steps which are known to be reversible are marked with opposing arrows, while all other steps are marked with a single arrow. See Discussion for a full explanation of the figure and each of the steps.

DISCUSSION

In this report we demonstrate the existence of cleavage events in the V(D)J recombination pathway, using both the *scid* mutation and a series of retroviral rearrangement cassettes with which individual steps could be assayed. Aberrant deletions associated with *scid* recombination events were observed on rearrangement templates containing a point mutation in one of a pair of signal sequences. These same templates, when introduced into wild-type pre-B cells, were inhibited in V(D)J recombination as scored by a Southern blot analysis. However, we observed junctional diversification without overall V-to-J rearrangement at the wild-type signal sequence/coding element border of these modified templates from both wild-type and *scid* pre-B cells. Introduction of a point mutation into both heptamer elements of the signal sequences eliminated the deletional phenotype of *scid* cells and the junctional diversification observed in wild-type cells. In addition, a retroviral template containing only one wild-type signal sequence did not show evidence of any type of V(D)J recombinational activity.

Intermediates in the V(D)J rearrangement pathway. A model of the progression of steps in V(D)J recombination which attempts to accommodate these new data is shown in Fig. 5. Evidence for most of these steps has been reviewed elsewhere (21). The first step involves recognition of the two signal sequences by the recombination machinery. Protein-protein interaction, rather than DNA-DNA pairing, are then hypothesized to bring the two signal sequence elements into close association, resulting in a protein-DNA complex (pairing). Site-specific cleavage, presumably staggered or blunt DSBs, at the junction of one of the coding elements and its signal sequence then occurs (one heptamer cleavage). Normally, the proposed reaction proceeds with cleavage at the second signal sequence (two heptamer cleavage) to generate an intermediate with four free DNA ends retained in close proximity to each other. DNA rearrangement occurs with the resorting of the four strands and the formation of two new junctions: the signal junction and the coding junction. The DNA sequences formed at these two junctions indicate that they are asymmetrically processed before being sealed. The coding junctions usually have processed ends (i.e., nucleotides added or deleted), and this junction is modified by one or more proteins involved in general DNA repair and recombination (coding junction [12]). Signal junctions, on the other hand, rarely show evidence of processing and so these ends may be joined by a V(D)J-mediated process (signal junction). This model effectively explains the types of recombination events observed using the JVD substrates.

A single, cleaved, signal sequence is a substrate for the *scid* mutation. Our results with mutated rearrangement cassettes in wild-type pre-B cells confirmed the previous findings that mutations in signal sequences inhibit V(D)J rearrangement (Table 1; 1, 15). Thus, we were surprised to observe deletional events in these mutated cassettes in *scid* cells (Fig. 2 and Table 1). This paradox between the *scid* and wild-type recombination phenotypes could be explained by distinguishing the origins of the *scid* deletions: either as products of aberrant rearrangement and coding junction formation or as products of single cleavage events.

When one heptamer in a pair of signal sequences is mutated, our experiments suggest that the cleavage process

can still proceed. DSBs stimulated by the recombinase complex cannot be directly measured because of the lack of an in vitro V(D)J recombination system. Instead, the evidence for this cleavage is shown by DNA sequence modifications, without overall V-to-J rearrangement, of the signal heptamer/coding element border (Fig. 4). Junctional diversification at wild-type signal sequences was observed for both wild-type and *scid* pre-B cells. These events could not be observed in NIH 3T3 fibroblasts containing the same constructs (Table 1 and data not shown). The sites of the base changes and the tissue specificity of the reaction indicate that the events are part of the V(D)J recombination mechanism. Thus, one heptamer cleavage (Fig. 5) may be an intermediate in V(D)J recombination.

The deletions in singly mutated templates were consistent with loss of the wild-type heptamer and not the mutant heptamer (Fig. 3). Also, we were unable to find any junctional diversification at mutated heptamer/coding element junctions by the PCR assay (data not shown). These experiments indicate that the cleavages are generated only at wild-type signal sequences. The consequences of this same cleavage event, however, will be different for recombination sites in wild-type versus *scid* cells. The primary outcomes are junctional diversification events in wild-type cells and deletional events in *scid* pre-B cells. Therefore, the *scid* phenotype can be detected following only a single DNA cleavage event mediated by V(D)J recombination.

Junctional diversification at a single recombination site has several features in common with coding junction formation. Our data indicates that both reactions were targets for *scid*, implicating the SCID protein in both steps normally. Also, base addition during junctional diversification occurred that had the features of N regions (Fig. 4). Base addition (N regions) was initially defined in coding junctions (3) and has been described previously with open/shut reactions (22). Finally, there is associated base deletion prior to the rejoining of DNA ends in both cases even though one of the flanking sequences in the open/shut reaction is a signal sequence that does not undergo base deletion in V(D)J rearrangement. Because of these common features, the enzymology of open/shut and coding junction formation is likely to be very similar.

Only certain DNA ends appear to be affected by the *scid* mutation. In addition to its role in V(D)J recombination, we have recently shown that the *scid* mutation affects generalized DSB repair following DNA damage (12). In this study, the majority of random DSBs introduced by X irradiation of *scid* fibroblasts were not repaired (12). Potentially, the double-stranded DNA (dsDNA) ends generated by X irradiation are subject to the same deletional phenotype observed with V(D)J recombination-induced breaks. These data compellingly suggest that the *scid* mutation can affect dsDNA ends not generated by V(D)J recombination.

However, not every dsDNA end may be a substrate for the *scid* mutant phenotype. Linear dsDNA transfected into *scid* pre-B cells was as stable as DNA transfected into wild-type pre-B cells (23). In addition, the two signal sequence ends that are joined to generate a signal junction are clearly less susceptible to the *scid* mutation than coding element ends (6, 9, 14, 23). However, aberrant deletions into the coding element as well as the signal element ends were observed during open/shut events in the presence of the *scid* mutation (Fig. 4C). These data suggest that the *scid* gene product does not selectively act on coding element ends per se, but may be dictated by the availability of any DNA end during the reaction. Thus, specific proteins may interact with certain DNA ends and render them refractory to the action of the *scid* mutation. For example, signal sequence ends may be sequestered by part of the V(D)J recombination complex, whereas the coding junction ends are accessible to the *scid*-mediated DNA repair pathway (Fig. 5).

The types, but not the frequency, of rearrangement products can be altered by signal sequence mutations. Three types of products can be formed in V(D)J recombination, depending upon whether a coding end is joined to (i) the other coding end, (ii) the other signal sequence in the pair (hybrid joints), or (iii) its own signal sequence (open/shut joints) (21). Normal coding junction formation is the predominant product in wild-type cells when both signal sequences are wild type, though hybrid joints can account for as much as 20% of the products (22). Mutation of one of a pair of signal sequences clearly alters the distribution of these products; hybrid joints and functional coding junctions could not be detected in either cell type.

Approximately 25% of the nonmutated JVD and DGR retroviral cassettes introduced into wild-type or *scid* pre-B cells rearrange (data not shown; 14). In *scid* pre-B cells, when V(D)J recombination is blocked by mutating one of the signal sequences, we still observed rearrangement of 29% of the cassettes (Table 1). Therefore, the overall frequency of recombination does not appear to be grossly affected by single mutations. A 100-fold reduction in the level of overall rearrangement with heptamer point mutations on extrachromosomal templates has previously been demonstrated (15). However, Hesse et al. (15) did not determine the effect of the heptamer point mutations on the open/shut process. Hence, the ability of cleavage events to initiate appears to be unaffected by mutations in the other signal sequence.

Approximately 4% of the singly mutated cassettes underwent unexpected deletions when introduced into wild-type pre-B cells. These events were V(D)J specific since the same viral templates introduced into fibroblast NIH 3T3 cells showed no evidence of rearrangement (Table 1), eliminating trivial explanations such as contaminating viral deletion variants. One possible origin of these events is a rearrangement between the wild-type signal sequence on the integrated retroviral template with a chromosomal signal sequence. This same type of rearrangement could presumably also account for some of the deletional events observed in *scid* cells. However, since deletions were observed eightfold more frequently in *scid* pre-B cells, a normal V(D)J rearrangement with chromosomal signal sequences cannot account for all of these aberrant rearrangements. In addition, we would have expected to see the same 4% frequency of deletions in wild-type pre-B cells containing the solo signal sequence construct, RV5-J_{solo}, if rearrangement to a chromosomal signal sequence could have occurred, whereas in fact no deletions were observed (Fig. 4B and Table 1). These results suggested that parameters such as retroviral integration sites and surrounding adventitious chromosomal signal sequences were not playing an important role in these experiments. One possible explanation for these data may be that these deletions are not entirely mediated by the normal V(D)J signaling pathway. Singly mutated cassettes block functional rearrangement and may therefore increase the availability of the resulting DSBs to participate in illegitimate recombination events leading to chromosomal deletions and translocations.

Pairing of signal sequences may be necessary for V(D)J recombination. V-to-J rearrangements and junctional diversification events were not detected in the retroviral cassette containing only a single signal sequence (Table 1). These

data suggest that cleavage of the RV5-J_{solo} signal sequence did not occur. However, as discussed above, cleavage of wild-type signal sequences readily occurred in the presence of a mutated signal sequence. These experiments indicate that interaction with a second signal sequence (even a mutated one) may be a necessary event for any V(D)J recombinational activity. These data further suggest that heptamer mutations may not disrupt the DNA-protein interactions necessary for the recombination complex to bind but can inhibit the ability of the complex to facilitate cleavage. The development of an in vitro system for V(D)J rearrangement will greatly facilitate the experimental testing of these ideas.

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