NORMAL RECOMBINATION SUBSTRATE V_H TO DJ_H REARRANGEMENTS IN PRE-B CELL LINES FROM SCID MICE

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Ig variable region genes are assembled from separate germline DNA gene segments during the early stages of pre-B lymphocyte development. The Ig heavy chain (H) variable region gene is first assembled from three (V_H, D, and J_H) gene segments. Subsequently, the Ig light chain gene (L) is assembled from two (V_L and J_L) gene segments. The site-specific recombination machinery (VDJ recombinase) that performs these joining events is apparently targeted by conserved signal sequences that flank all variable gene segments (see references 1, 2 for a review). VDJ recombinase mediated rearrangements have been proposed to occur by a nonreciprocal mechanism in which the coding and signal sequences are differentially processed. Joining of the coding sequences (coding join) is often imprecise; the imprecision results from potential deletions of several base pairs (bp) that can affect one or both fused sequences and/or from addition of extra bases at the junction (N regions) (1). Nucleotides are rarely lost from the fused signal sequences (signal join), although extra nucleotides may be added (3, 4).

Mice homozygous for the *scid* mutation are impaired in their ability to assemble functional antigen receptor variable region genes, and as a result, they have an inability to generate functional B and T lymphocytes (5). Abelson murine leukemia virus (A-MuLV)[1]-transformed pre-B cell lines (6) derived from mice homozygous for the *scid* mutation exhibit a profound alteration of the VDJ recombination process (7-11). Studies of these lines indicated that the VDJ recombinase in scid mice pre-B cells can recognize appropriate gene segments and make appropriate endonucleolytic scissions between coding and signal sequences; the defect appears to affect the terminal stages of coding joining formation at the point when the ends are to be ligated. The aberrant scid coding rearrangements usually result either in

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[1] *Abbreviations used in this paper:* A-MuLV, Abelson murine leukemia Virus; PCR, polymerase chain reaction.

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joining of one segment to sequences distal to the other ("normal" joins with respect to one partner) or in recombination between sequences distal to both segments (large deletions) (7, 9). Although this defect abrogates ability to efficiently join coding segment ends, joining of signal segment ends is relatively unaffected (9-13). The large deletions observed in attempted coding joins of endogenous IgH and IgL rearrangements in A-MuLV transformed scid pre-B cells were proposed to result from an illegitimate recombination event that maintains chromosomal integrity during an abortive attempt at coding joins formation (9, 12, 14). These aberrant recombination events are presumed to occur through a non-VDJ recombinase-mediated mechanism (9).

Approximately 15% of scid mice eventually develop a "leaky" phenotype characterized in part by an oligoclonal expansion of B cells and the appearance of serum Ig (15). "Leakiness" could result from the reversion of the *scid* defect in clones of developing B (T) cells (or stem cells) and/or from the occurrence of "normal" recombination events at low frequency in scid pre-B cells. To test whether scid pre-B cells are capable of forming "normal" coding joins at detectable frequency, we introduced into A-MuLV scid cell lines a recombination substrate in which V_H to DJ_H joining activates a drug-selectable gene (*gpt*).

Materials and Methods

Cell Lines. Derivation, cell culture, and characteristics of A-MuLV-transformed normal 38B9 and 300-18P cells, and scid SC7, SC24, and SC44 cell lines have been previously described (1, 9, 16). Isolation of *neo*- and *gpt*-expressing cell populations and subcloning by limiting dilution were as described (17).

Recombination Substrates. Construction of the RV-DJ_H recombination substrate, production of retrovirus, lymphocyte infection, analysis of the integrated RV-DJ_H construct by Southern blotting, and characteristics of the pM7 probe have been previously described (17).

Transfection Experiments. Transfections of the pJH195 recombination substrate into A-MuLV cell lines, rescue of the introduced plasmid DNA, transformation of DH5 bacteria and selection on ampicillin and ampicillin + chloramphenicol-supplemented media were as described (13, 18).

PCR Assays. PCR analyses were performed according to Saiki and collaborators (19) using a Perkin Elmer Corp. (Pomona, CA) Thermocycler. Oligonucleotide 1 is a 27-mer specific for MSV-MuLV retroviral sequences; oligonucleotides 2 and 3 are 27-mers specific for 5' and 3' gpt gene sequences, respectively; oligonucleotide 4 is a 30-mer specific for a noncoding sequence flanking the 3' side of the J_H3 gene segment.

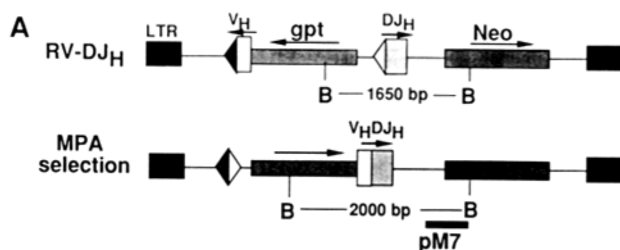
Cloning and Sequencing. For cloning purposes, PCR reactions were spun through Centricon 30 columns (Amicon Corp., Danvers, MA) and ligated into the Bam HI site of M13-mp18 or Bluescript vectors (Stratagene, La Jolla, CA). Nucleotide sequencing was performed by the Sanger dideoxy protocol.

Results

Formation of Coding Joins in A-MuLV scid Pre-B Cells. The A-MuLV-transformed scid pre-B cells SC7, SC24, and SC44 express RNA transcripts characteristic of early pre-B cells and rearrange the IgH locus in culture, implying that they have an active VDJ recombinase system (9, 12, 14). However, various lines of evidence indicate that assembly of functional V_HDJ_H regions in these cells is either nonexistent or very infrequent (14). Accordingly, all of the multiple characterized endogenous J_H-associated rearrangements in these three lines were grossly abnormal (as outlined above) (9). To further define their recombination defect, we assayed for the ability

of these cell lines to rearrange the retrovirus-based recombination substrate RV-DJ_H (17). As previously described (17), this construct contains a constitutively expressed neomycin-resistance gene that allows selection of cells in which it is integrated. In addition, the construct contains a bacterial *gpt* gene, inserted between the oppositely oriented V_H and DJ_H variable segments that is only correctly expressed (from the retroviral LTR promotor) following its inversion through a VDJ recombinase-mediated V_H to DJ_H joining (Fig. 1 *A*). Thus, the gpt gene allows for selection of the cells having undergone such inverted rearrangements. This "inversion" recombination substrate retains products of both normal coding and signal joins mediated by VDJ recombinase activity (Fig. 1 *A*). We will refer to cells carrying the RV-DJ_H substrate and selected for the *neo* or *gpt* activity as RV-DJ_Hneo or gpt, respectively.

Rearrangements within the RV-DJ_H substrate can be monitored by Southern blotting: in particular, the presence in Bgl II-digested genomic DNA of a 2.0-kb fragment, instead of the unrearranged 1.65-kb fragment, hybridizing with the pM7 probe is diagnostic for a correct inverted rearrangement (reference 8 and Fig. 1 *A*). As described previously, fragments corresponding to such inverted rearrangements are readily detected in the DNA from a normal pre-B line (e.g., 38B9RV-DJ_Hgpt cells; Fig. 1 *B*, lane *9*). Note that submolar amounts of the same fragment are also present in the DNA from 38B9RV-DJ_Hneo cell population (Fig. 1 *B*, lane *8*), showing that substrate rearrangements can be detected before selection for *gpt* activity in normal



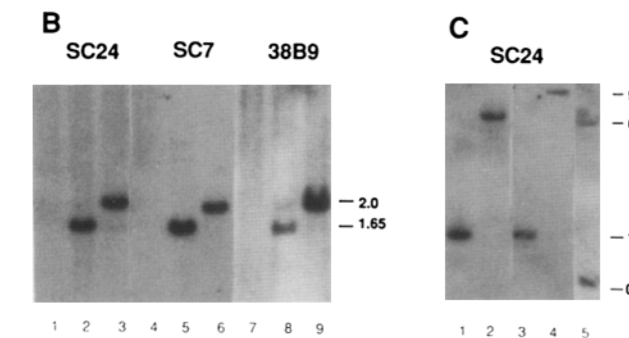


FIGURE 1. (*A*) Partial restriction maps of the RV-DJ_H inversion substrate and of the VDJ recombinase-mediated recombination product. The retroviral LTRs are indicated by black boxes, the *neo* and *gpt* genes by shaded boxes, the signal sequences flanking the V_H81X and DJ_H3 segments by black (23 bp spacer) or open (12 bp spacer) triangles, respectively. Arrows indicate the direction of transcription within these various elements (none of these elements are drawn to scale). The position of the pM7 probe used for the Southern analyses is indicated below the maps. (*B* and *C*) Southern blotting analysis of the RV-DJ_H retrovirus in scid (SC24 and SC7) and in normal (38B9) A-MuLV transformed cells. (*B*) lanes *1*, *2*, *3*, respectively; DNA from SC24, SC24RV-DJ_Hneo, and SC24RV-DJ_Hgpt cell population; lanes *4*, *5*, *6*, respectively; DNA from SC7, SC7RV-DJ_Hneo, and SC7RV-DJ_Hgpt cell population; lanes *7*, *8*, *9*, respectively; DNA from 38B9, 38B9RV-DJ_Hneo, and 38B9RV-DJ_Hgpt cell population. (*C*) lanes *1* to *5*: DNA from five subclones (obtained by limiting dilutions) of the SC24RV-DJ_Hneo cell population. The positions of the unrearranged (1.65 kb) and rearranged (2.0 kb: normal rearrangements; 9.2 kb, 6.6 kb, and 0.8 kb: aberrant rearrangements; see text) pM7 hybridizing Bgl II fragments are indicated on the right.

pre-B cells that express high levels of VDJ recombinase activity. In contrast, SC7-, SC24-, and SC44RV-DJ_Hneo genomic DNAs contain, at detectable level, only the unrearranged (1.65 kb) pM7-hybridizing Bgl II fragment (Fig. 1 *B*, lanes *2* and *5*; only SC24- and SC7RV-DJ_Hneo are shown). On longer exposures, DNA from the scid pre-B lines eventually exhibit additional hybridizing fragments of aberrant size (data not shown), suggesting that the introduced construct can be rearranged in scid pre-B cell lines. Accordingly, Bgl II–digested DNA from subclones of neo-selected scid pre-B cell lines either retained the unrearranged construct band or contained hybridizing fragments that were clearly aberrant in nature since they differed in size from the 2.0-kb diagnostic fragment (Fig. 1 *C*; DNA from five SC24RV-DJ_Hneo cell subclones are shown). These results suggest that attempts made by the scid cells to rearrange inversion substrates generally result in aberrant joining events. Although we did not determine the precise structure of the modified RV-DJ_H copies in SC24RV-DJ_Hneo cell subclones, some appear to involve deletion events on the 5′ side of the substrate because polymerase chain reactions (PCR; reference 19) using genomic DNA from such subclones and various sets of oligonucleotides specific for this region of the substrate generally failed to amplify any DNA fragment (data not shown).

The efficiency of *gpt* selection varied among the scid RV-DJ_H cell lines. For example, the resistant cells appeared much more slowly within the selected SC7-RVDJ_Hgpt as opposed to the SC24- and SC44-RV-DJ_Hgpt cell populations. Notably, the appearance of actively growing cells within the *gpt*-selected SC24- and SC44RV-DJ_H cells was not markedly delayed in comparison to that observed for normal 38B9RV-DJ_H cells. These differences might reflect different percentages of cells expressing VDJ recombinase activity and/or different levels of this activity in the cell lines tested. Surprisingly, genomic DNAs from all *gpt*-selected scid populations contained a unique pM7-hybridizing fragment with a size compatible with correct inverted rearrangements (SC24RV-DJ_Hgpt and SC7RV-DJ_Hgpt hybridization patterns are shown on Fig. 1 *B*, lanes *3*, *6*; an identical result was obtained with SC44RV-DJ_Hgpt cell DNA, data not shown). To analyze more precisely these apparently normal rearrangements, we used the PCR technique to amplify specific DNA regions of the RV-DJ_H construct. According to the strategy depicted in Fig. 2 *A*, PCR assays using various sets of RV-DJ_H–specific oligonucleotides should amplify different DNA fragments of predictable size from genomic DNA containing a correctly inverted V_H to DJ_H rearranged copy of the RV-DJ_H substrate. They include a fragment of ~650 bp containing the signal join (oligonucleotides 1+2) and a fragment of ~560 bp containing the coding join (oligonucleotides 3+4). Fragments of approximately the expected size were effectively amplified from all SCRV-DJ_Hgpt, but not neo, selected cells (data not shown). Together, these results strongly suggest that the *gpt* selection allows isolation of scid pre-B cells that have undergone correct inversion rearrangements within the introduced RV-DJ_H recombination substrate.

The amplified DNA products from DNA of 38B9 and scid RV-DJ_Hgpt pre-B lines were molecularly cloned and the nucleotide sequences of the joint regions were determined. For this purpose, we also used a combination of oligonucleotides 1+4 to clone DNA products (~1,900 bp in length) that should contain (on the same DNA fragment) both signal *and* coding joins, if amplified from the inverted substrate (see

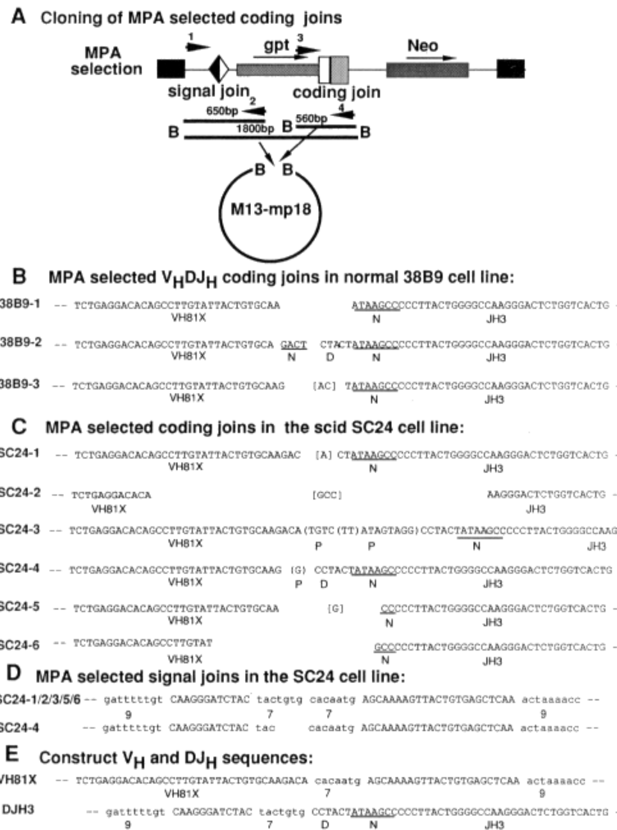


FIGURE 2. Structure of RV-DJ_H Rearrangements. (*A*) Strategy to analyze RV-DJ_H rearrangements by PCR and to molecularly clone the DNA fragments containing the signal and coding joins from the inverted retroviral substrate. The positions of the RV-DJ_H specific oligonucleotides used in the PCR reactions (oligonucleotides 1+2, 3+4, and 1+4) and in the sequencing reactions to analyze the nucleotide sequence of the signal or coding joins (oligonucleotides 1 and 3, respectively) are indicated (the oligonucleotides and the various elements of the substrate are not drawn to scale, B indicates the Bam HI restriction sites included at the 5' side of the oligonucleotides and used to subclone the PCR products into the vectors M13-mp18 or Bluescript (Stratagene, La Jolla, Cal.). (*B*, *C*, *D*) Nucleotide sequences of coding (*B*, *C*) and signal (*D*) joins within the RV-DJ_H construct in 38B9RV-DJ_Hgpt (*B*) and SC24RV-DJ_Hgpt (*C*, *D*) cells. All sequenced products were amplified with oligonucleotides 1+4, except 38B9-1 and 38B9-2, which were amplified with oligonucleotides 3+4. (*E*) Partial nucleotide sequences of the V_H and DJ_H fragments used in the RV-DJ_H substrate. N regions are underlined. *P* regions are shown in parenthesis and noted. The joins in *B*, *C*, and *D* have been aligned with the sequences in *E* so that base loss is apparent; bases that could be derived from either partner in the rearrangements of panels *B* and *C* are shown between brackets. In *D* and *E*, heptamers and nonamers of the signal sequences are indicated by lower case letters.

Fig. 2 *A*). In Fig. 2, the DNA sequences spanning the joining site of three different PCR products from 38B9 cells (panel *B*) and six different PCR products from SC24 cells (panels *C* and *D*) are compared with the sequences of the unrearranged V_H81X and DJ_H3 segments used in the construct (panel *E*). Confirming the Southern and PCR analyses (see above), several SC24 coding joins appear normal with loss of a few nucleotides on one or both partners (for example SC24-1, SC24-4, and SC24-5). Both SC24-3 and SC24-4 coding joins contain extra nucleotides that are characteristic of P nucleotide regions (20); it is interesting to note that the P regions in SC24-3 form in both the V and DJ sides, longer palindromes than the usual tetranucleotide palindrome (20).

In the SC24-6 and SC24-2 coding joins the number of bases that have been deleted from one or both rearranged ends, respectively, is greater than usually observed in normal V_HDJ_H joins (reference 21; also compare with panel *B*). However, these rear-

rangements still involve both V_H *and* J_H coding sequences and, thus, are not grossly abnormal like scid endogenous Ig joins previously reported (7-12). Since the nucleotide sequence analysis of one PCR product from each SC7- and SC44RV-DJ_Hgpt genomic DNA also showed similar correct coding joins (the SC7 join has a 2-bp N region and a 4-bp deletion on the D side [14], the SC44 join has 1-bp and 5-bp deletions on the V and D side, respectively, data not shown), we conclude that the *gpt* selection allows for the isolation of scid cells that have carried out relatively normal V_H to DJ_H joins within the introduced inversion construct. All six amplified products from SC24RV-DJ_Hgpt cell DNAs but one contain signal joins consisting of the precise back-to-back fusion (at the heptamer coding borders) of the V_H and DJ_H flanking signal sequences (panel *D*). The exception, the signal join in SC24-4 (which has a normal coding join, see above), has a deletion of the four D proximal (TGTG) nucleotides from the DJ_H3 heptamer.

The scid Defect Is Not Corrected in the gpt-selected Cells. Normal coding joins within recombination substrates introduced into A-MuLV scid cell lines were not observed by others who used an extrachromosomal, nonselectable, recombination assay (13). We used this assay to test the possibility that the selection for gpt activity resulted in the isolation of revertant cells that have corrected the *scid* mutation. For that purpose, both *neo*- and *gpt*-selected SC24 cell populations were transfected with the recombination substrate pJH195 that has been previously used to analyze VDJ recombinase activity in various normal and scid A-MuLV cell lines (13, 18). In normal A-MuLV cell lines, VDJ recombinase-mediated deletion and/or inversion rearrangement events within the transiently transfected pJH195 construct allow the rescued rearranged plasmid DNA to confer chloramphenicol resistance to bacteria (see reference 18 for details). In accord with previous findings (13), recovery of chloramphenicol-resistant plasmids are reduced in scid pre-B cells compared with a normal A-MuLV pre-B cell line (Table I). However, the reduction affected both *neo*- and *gpt*-selected scid cells. In addition, the structures of most of the chloramphenicol-rescued plasmids are equally grossly altered after transfection into both selected scid cell populations, whereas all the analyzed recombinants from the normal 300-18P pre-B cells exhibited a correctly rearranged profile (see Table I, legend for details). Thus, the recombination abilities of the SC24RV-DJ_Hneo and SC24RV-DJ_Hgpt cell populations are not significantly different when tested with the extrachromosomal pJH195 recombination substrate. This finding indicates that the *scid* defect has not been corrected in a majority of the cells in the SC24RV-DJ_Hgpt cell population although the RV-DJ_H substrate is correctly rearranged in most of these cells (see above).

Discussion

The RV-DJ_H rearrangements that we detected in the scid RV-DJ_Hgpt cell populations may give insight into the nature of the molecular events responsible for scid leakiness. The most frequent recombination event in scid lines is the grossly aberrant rearrangement of the RV-DJ_H substrate (involving sequences away from the V and DJ coding regions). This rearrangement is observed in subclones of *neo*-selected populations (i.e., not selected for recombination events) and appears to be analogous to the aberrant rearrangements observed at endogenous scid Ig loci. In con-

TABLE I
Rearrangements of Extrachromosomal Substrate pJH195

Cell line*	RepAmp‡	RepAmpCam§	R‖	Structure¶
300.18p	374	134	0.36	#10 [10 +]
SC24 Neo	257	18	0.07	#15 [13 – /2 +]
SC24 gpt	128	8	0.06	#8 [7 – /1 +]

* The substrate pJH195 was transfected into the indicated A-MuLV pre-B cell lines, recovered after 48 h, and used to transform DH5 bacteria that were selected on ampicillin or on ampicillin + chloramphenicol supplemented media, as described (12); 300.18p is an A-MuLV transformed cell line from a normal mouse.

‡,§ Number of recombinant colonies growing on ampicillin and ampicillin + chloramphenicol, respectively. The results of two experiments are combined.

‖ Frequency of recombination after transfection into the corresponding cell lines calculated as the ratio between the number of recombinants obtained in ‡ and *, respectively.

¶ The structure of the rescued recombinants was determined by enzyme restriction digests (Sal I and Pvu II) on DNA minipreps and analysis on agarose gels. # indicates the number of recombinants analyzed. +, indicates the number of recombinants with digest profiles that agree with a correct rearrangement. –, indicates the number of recombinants with aberrant profiles. Among the rearrangements rescued from the 300.18p cells, Pvu II digests suggest that eight result from deletions and two result from inversions; the same Pvu II restriction digests suggest that the + recombinants rescued after transfection into SC24 Neo and SC24 gpt result from normal signal joinings that occurred at the A site of this particular construct (see reference 12 for details).

trast, when scid pre-B lines are selected for expression of the *gpt* gene, we observe relatively normal joins within the RV-DJ_H substrate (involving both coding regions). These normal joining events in scid pre-B lines must occur at significantly lower frequency than the aberrant joining events based on the inability to observe them in unselected scid populations or their subclones (this study and references 7–10, 12). Furthermore, the formation of relatively normal coding joins that we observed within the selectable, integrated inversion substrate was not observed with transient recombination substrates (reference 13 and this study). This discrepancy might reflect intrinsic differences between the two recombination substrate systems (such as structure or stably integrated versus extra-chromosomal recombination) or from selection differences (see below) between the two systems that lead to the preferential recovery of RV-DJ_Hgpt cells that have formed relatively normal rearrangements. Thus, our ability to observe the normal joins after selection for the inversional activation of the gpt gene may result, at least in part (see below) from the requirement to maintain integrity of the gpt gene.

Although the *gpt* selection pressure applied to the RV-DJ_H construct should limit extensive coding join deletions, the 5′ V_H sequences (total of 210 bp) and/or DJ_H 3′ flanking sequences (total of 235 bp) could theoretically be deleted during coding join formation without affecting the *neo*/*gpt* genes within the retroviral substrate. However, our analyses suggest that the range of deletion remains extremely restricted in *gpt*-expressing cells. In particular, of a total of eight amplified coding join rearrangements, six had limited deletions within a range also observed in a normal 38B9 pre B cell line (Fig. 2 *B*). These results could be explained by assuming that the

scid mutation does not entirely abolish normal VDJ recombinase activity, but rather affects this activity in a way such that it becomes highly inefficient. According to this explanation, scid pre-B cells would express normal VDJ activity, but at a low level compared with the aberrant activity. In this context, the selection for inversion events that maintain integrity of the *gpt* gene might lead to selective recovery of the normal events. However, we cannot rule out the possibility that an alternative mechanism (as outlined above) also leads to an illegitimate recombination event that resolves VDJ recombinase-mediated chromosomal breaks near both V_H and DJ_H coding ends to form "normal" VDJ joins. This possibility could explain the finding that two of eight scid RV-DJ_H joins had relatively large deletions (beyond that usually observed in normal coding joins). Finally, it should also be noted that these two mechanisms are not mutually exclusive.

The "normal" rearrangement events that we observe in the recombination substrate should also occur in endogenous genes of developing scid precursor lymphocytes and, thus, may explain leakiness. Two separate "normal" recombination events are required to assemble a functional heavy chain variable region gene (D to J_H and V_H to DJ_H) and one to assemble a functional light chain variable region gene (V_L to J_L); a similar situation exists for developing T cells. Thus, given the requirement for three different normal joins and the probability that normal joins are formed at least several orders of magnitude less efficiently than aberrant joins in scid pre-B cells (7-10, 12), functional lymphocytes would be generated as much as 10^6 times less efficiently (or more) as in normal mice. Given the large number of lymphocytes generated in mice each day (22), such an efficiency could conceivably generate the normal lymphocytes observed in leaky scid mice. However, our results do not exclude the possibility that leakiness also could result from a reversion of the *scid* mutation in a given differentiating precursor lymphocyte (23). Because the RV-DJ_Hgpt cell population cannot correctly rearrange a secondary transfected DNA (see above), reversion clearly is not the mechanism responsible for the majority of the normal RV-DJ_H substrate rearrangements (requiring only one joining event) that we detected in scid pre-B lines. However, if the *scid* mutation were a point mutation, it is conceivable that reversion events (which would then permit all subsequent joins to be normal) may occur as frequently (or more frequently) than the multiple "normal" joining events in a single cell required to generate a functional lymphocyte in the presence of the *scid* defect.

Summary

To further analyze the VDJ recombination defect in lymphoid pre-B cells from mice with severe combined immune deficiency (scid mice), we have assayed the ability of Abelson murine leukemia virus (A-MuLV) transformed pre-B cells from scid mice to rearrange a recombination substrate in which inverted V_H to DJ_H joins activate a selectable (*gpt*) gene. In unselected populations, substrate rearrangements occurred frequently, but were aberrant and probably analogous to the aberrant rearrangements observed at endogenous scid Ig gene loci. In contrast, populations of scid pre-B lines selected for *gpt* activity within the substrate contained mostly "normal" V_H to DJ_H joins within the introduced substrate. These findings demonstrate that scid pre-B cells can make normal joins at low efficiency and are discussed with re-

spect to the potential mechanism of the scid defect and the occurrence of Igs in leaky scid mice.

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