High Frequency of Normal DJ_H Joints in B Cell Progenitors in Severe Combined Immunodeficiency Mice

By Jacqueline L. Pennycook, Yenhui Chang, Jakub Celler, Robert A. Phillips,* and Gillian E. Wu

*From the Department of Immunology and *The Hospital for Sick Children Research Institute, University of Toronto, Toronto, Ontario, Canada, M5S 1A8*

Summary

The severe combined immunodeficiency (*scid*) mouse has a defective V(D)J recombinase activity that results in arrested lymphoid development at the pro-B cell stage in the B lineage. The defect is not absolute and *scid* mice do attempt gene rearrangement. Indeed, ~15% of all *scid* mice develop detectable levels of oligoclonal serum immunoglobulin and T cell activity. To gain more insight into the *scid* defect and its effect on V(D)J rearrangement, we analyzed DJ_H recombination in *scid* bone marrow. We determined that DJ_H structures are present in *scid* bone marrow and occur at a frequency only 10–100 times less than C.B-17+/+. The *scid* DJ_H repertoire is limited and resembles fetal liver DJ_H junctions, with few N insertions and predominant usage of reading frame 1. Moreover, 70% of the DJ_H structures were potentially productive, indicating that normal V(D)J recombinants should be arising continually.

The formation of functional Ig and TCR genes requires the rearrangement of several genetic elements encoding the variable regions: V, J, and, in some cases, D (1). The mechanism of this recombination is only now beginning to be elucidated, and most of what we know about the process is derived from studies of its substrates and its products (for review, see references 2 and 3). The process of rearrangement is mediated by an enzymatic system, the V(D)J recombinase, which is targeted by the recombination signal sequences (RSS)[1] flanking the elements to be rearranged. RSS consist of three parts; a heptamer, a 12- or 23-bp spacer, and a nonamer. As a result of V(D)J recombination, two types of junctions are formed: signal joints and coding joints (4). In the signal joints, which are usually precise, RSS are joined in a head-to-head orientation. Coding joints, which are usually imprecise, have a few nucleotides added and/or deleted at the coding termini.

V(D)J recombination is essential for normal lymphoid development. Mice deficient for the recombinase activating genes, Rag-1 and Rag-2, as well as *scid* mice, have defective V(D)J recombinase activity (5–8), and lymphoid development in all these mice is arrested at the pro-B cell stage. Unlike the Rag-1$^-$ and Rag-2$^-$ mice, however, *scid* mice attempt gene rearrangement, but these attempts fail to produce functional receptors. In Abelson murine leukemia virus (A-MuLV)-transformed *scid* bone marrow lines (5, 9) and in long-term cultures of *scid* bone marrow (10), there are large deletions in the coding joints even though signal joints are relatively normal (8, 11).

The *scid* defect is leaky. Approximately 15% of all *scid* mice develop detectable levels of oligoclonal serum Ig and T cell activity (12–15). The incidence of leakiness varies with both the age of the mice and their environment (12). Petrini et al. (16) have postulated that a genetic reversion occurs in a B cell precursor permitting normal V(D)J recombination in subsequent daughter cells. In support of this hypothesis, some functional T cell clones derived from leaky *scid* mice have normal rearrangements on their nonexpressed TCR alleles. This result is unexpected from the high frequency of abnormal coding joints found in A-MuLV *scid* lines (16, 17). Further, normal TCR rearrangement at the δ DJ locus has been observed in *scid* thymocytes (18, 19). However, there is no evidence of attempted Vδ rearrangement or rearrangement at the β and γ loci (20). An alternative hypothesis to explain the leaky phenotype is that *scid* mice make productive rearrangements at a higher frequency than previously expected (see below), so that a few B cells are produced each day depending on the chance occurrence of several rearrangement events. Thus, whether or not a mouse becomes leaky depends in part on whether a chance clone of B cells is stimulated by antigen before it is eliminated as part of the normal B cell turn-over.

[1] *Abbreviations used in this paper*: A-MuLV, Abelson murine leukemia virus; BM, bone marrow; RF, reading frame; RSS, recombination signal sequence.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/09/1007/10 $2.00
Volume 178 September 1993 1007–1016

To gain more insight into *scid* defect and its effect on the mechanism of V(D)J rearrangement, we have analyzed DJ_H recombination products in *scid* mice. Using a quantitative PCR assay devised to determine the extent of DJ_H formation (21), we found DJ_H structures in *scid* mice occur at a frequency only 10–100 times less than in C.B-17+/+ mice. Sequencing of these structures revealed that the *scid* DJ_H repertoire is limited and resembles fetal liver DJ_H junctions, with few N insertions and predominant usage of reading frame 1. Moreover, 70% of these structures were potentially productive, indicating that normal V(D)J recombinants should be arising continually in *scid* mice.

Materials and Methods

Mice and Cell Lines. The C.B-17*scid* (referred to in the text as *scid* mice) and C.B-17 wild type (referred to in the text as C.B-17+/+ mice) were originally obtained from Melvin Bosma (Fox Chase Cancer Center, Philadelphia, PA). C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mouse strains were bred and maintained in the animal colony of the Ontario Cancer Institute (Toronto, Canada) under defined flora conditions. Nonleaky and leaky *scid* mice were differentiated on the basis of serum Ig detected by Ouchterlony diffusion. Bone marrow (BM) and spleens were removed from individual mice at 6 or 27 wk of age. A-MuLV cell lines were derived and maintained as described (22).

DNA Preparation. Single-cell suspensions were prepared from the adult BM and spleens using standard procedures (23). Genomic DNA was isolated from the BM, spleen, and cultured cell lines by the "spooling" method as previously described (22).

Oligonucleotide PCR Primers. The DFS primer is a 31mer hybridizing to the 5′ RSS of the D_H elements of both the Dsp and the DFL families. Its sequence is 5′-AGGGATCCTTGTGAAGGGATCTACTACTGTG-3′. The DQ52 primer is 5′-GCGGAGCACCACAGTGCAACTGGGAC-3′. It is a 26mer specific for DQ52; it hybridizes to the region within the 5′ spacer through the heptamer and contains all of the DQ52 coding region. The J_H4 primer is 5′-AAAGACCTGCAGAGGCCATTCTTACC-3′. It is a 26mer containing sequences of the major J-C intron immediately 3′ of J_H4. The J_H2-IN primer is 5′-TGGCCAGGATCCCTATAAATCTCTGG-3′. It is a 26mer that contains sequences ~200 bp 3′ of J_H2 in the J_H2-J_H3 intergenic sequence. The J_H4-IN primer is 5′-GAGGAGACGGTGACTGAGGTTCCTTG-3′. It is a 26mer that is entirely contained within the J_H element and shares no overlapping sequence with the J_H4 primer (described above). The oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) and purified using NENSORB PREP cartridges (Du Pont Co., Wilmington, DE).

Standard PCR Assay. PCR amplifications were performed as described (21). Fresh aliquots of reagents were used for each set of experiments. In brief, a set of standard DNAs derived from A-MuLV lines containing known numbers of DJ_H targets were diluted into DNA that had no DJ_H targets (CB32, a VDJ/VDJ A-MuLV line). The diluted standards were amplified at the same time as the experimental and control DNAs. A hot start method with Ampliwax (Perkin-Elmer, Emeryville, CA) was used, with the Taq polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN) being added when the reaction temperature reached 80°C. 30 cycles were carried out. To facilitate the cloning of the larger DJ_H structures (DJ_H1 and DJ_H2), secondary amplifications were performed on 10- and 100-fold diluted primary *scid* and C.B-17+/+ amplifications, respectively; one of the nested primers (J_H2-IN or J_H4-IN) was used with the same cycling conditions.

Southern Hybridization Analysis. 10% of each PCR amplification reaction was loaded on a 1% agarose gel (Sigma Chemical Co., St. Louis, MO) and electrophoresis in TAE buffer (23). Gels were Southern blotted on Zeta Probe nylon membrane (Bio-Rad Laboratories, Richmond, CA) using a vacuum blotting system (Vacugene; LKB Pharmacia). Filters were probed with a ^{32}P-labeled J_H4 probe under conditions previously described (24). Autoradiographs were exposed without intensifying screens either with Kodak film or in a phosphorimager cassette.

Densitometry. Densitometry was used to measure the relative intensities of bands on the autoradiographs. Several exposures of Southern blots were scanned using a densitometer (Molecular Dynamics, Sunnyvale, CA). An example of the raw data output for one analysis is shown in Table 1. The data were entered in an Macintosh Excel data base and the number of DJ_H structures was calculated using the TREND function based on the curves generated by the standard values. The averages of a number of experiments and their standard errors were determined using StatviewII. The values obtained were per microgram of DNA. These values were converted (Table 2) into numbers per organ using the factors: 1 μg of DNA is 1.8×10^5 haploid genome equivalents; 2×10^7 nucleated cells are in a femur; 1.5×10^8 nucleated cells are in a C.B-17+/+ spleen; and 3×10^7 nucleated cells are in a *scid* spleen (25). At times the data from the C.B-17+/+ mice lay outside the standard curve. These estimates were necessarily less accurate.

Cloning and Sequencing of DJ_H Products. Products derived from the DFS/J_H primer pair were cloned from amplifications separate from those used for quantification. To prevent contamination, *scid* and C.B-17+/+ DNA were not amplified at the same time, nor were their amplified products run on the same gel. Negative controls were included with all rounds of amplification. These controls consisted of no DNA and targetless DNA from the 70Z/3 cell line. 70Z/3 has a VDJ and a DQ52/J_H2 rearrangement, and thus cannot be amplified with the DFS/J_H primers. Some primary C.B-17+/+ and *scid* amplifications were cloned directly, and others were cloned after secondary amplification. In these cases, either secondary amplification proceeded directly or primary amplified samples were run on lo-melt gels (Nusieve), and plugs, taken from the control and experimental lanes, were amplified in parallel using nested primers. Whenever a product was detected in the negative control lanes, the amplifications were discarded.

The amplified structures were cloned by means of the TA Cloning Kit (Invitrogen) or by the embedded restriction endonuclease sites in the PCR primers. Bacteria harboring positive plasmids were identified by antibiotic selection. Nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) bearing bacterial DNA were hybridized at 42°C or 37°C overnight with DIG-dUTP-labeled (Biochemica; Boehringer Mannheim Biochemicals) J_H2 or J_H4 probes, respectively. The filters were washed twice with 2× SSC, 0.1% SDS for 5-min intervals followed by two 1× SSC, 0.1% SDS washes, at 60°C or 42°C for 30 min. The reduced hybridization and washing temperatures for the J_H4 probe allowed detection of the 50-nucleotide homology in the DJ_H4 structure. To detect the signal, filters were blocked in a solution of 5× SSC, 5% skim milk solution, 50% formamide, 0.1% *N*-lauryl sarcosyl, and 0.02% SDS for 3 h and then exposed to an anti-DIG horseradish peroxidase (POD)-conjugated antibody (Biochemica; Boehringer Mannheim Biochemicals) diluted 1/1,000 in blocking solution for 1 h. The filters were washed in 100 mM Tris, pH 7.5, 150 mM NaCl, and 0.02% Tween20 four times for 15 min each, and then subjected to the enhanced chemiluminescence (ECL) detection

system (Amersham Corp., Arlington Heights, IL). Positive colonies were selected and plasmid DNA was extracted. Sequencing was performed using the double-stranded method with the T7 Sequencing Kit (Pharmacia Fine Chemicals, Piscataway, NJ); both the reverse and universal sequencing primers were utilized.

Results

Quantification of DJ_H Structures. To quantitatively assay DJ_H structures by PCR we used primers flanking D and J_H4, which results in a "ladder" of DJ_H1, DJ_H2, DJ_H3, and DJ_H4 when the products are analyzed by gel electrophoresis (Fig. 1 *a*). By simultaneously amplifying standards containing equimolar concentrations of targets of each of the four DJ_H products, we can estimate their number in tissues. We previously showed that none of these products are preferentially amplified by the DFS/J_H4 primers (21). Fig. 1, *b* and *c*, show examples of typical PCR amplifications of DJ_H rearrangements in BM and spleen DNA from individual *scid* and C.B-17+/+ mice. There are four discrete bands of sizes corresponding to DJ_H1, DJ_H2, DJ_H3, and DJ_H4 structures in all samples. The discrete nature of the bands was somewhat surprising due to the paucity and aberrant nature of the *scid* coding junctions found previously (5, 11). We quantified these products using data derived from seven C.B-17+/+ BM, five C.B-17+/+ spleens, five *scid* BM, and four *scid* spleens. One of the scids was serum Ig positive. The raw data from a typical quantification are shown in Table 1; a summary of the quantification data is shown in Table 2. The data are presented as the sum of the four J_H's because in some cases individual *scid* mice had unequal usage of the J_H segments (Fig. 1, *b* and *c*, and Discussion). This unequal usage of J_H was not observed in other

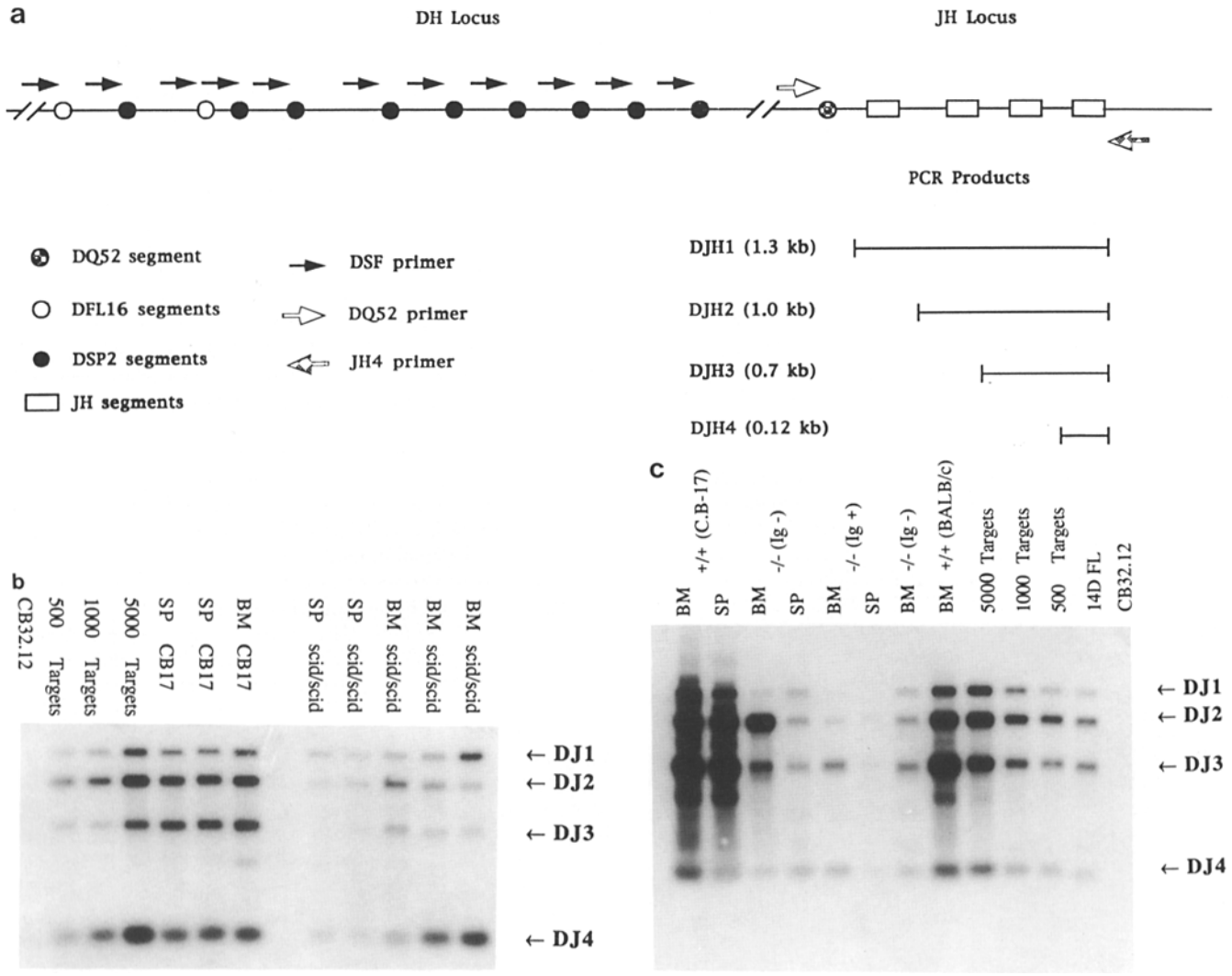

Figure 1. (*a*) Genomic map of the DJ_H locus showing oligomer primer position sites and expected products (20), not drawn to scale. (*b*) Southern blot analysis of PCR amplification of *scid* and C.B-17+/+ BM and spleen (*SP*) DNA using the DFS/J_H4 primer pair. Amplifications of the titration standards were run in parallel. (*c*) Southern blot analysis of PCR-amplified BM and SP DNA from nonleaky (Ig^-) and leaky (Ig^+) *scid* and C.B-17+/+ mice. 14D FL is 14-d C57BL/6 fetal liver DNA amplified for comparison (see reference 21), CB32.12 is a negative control. Titration standards were amplified in parallel as in *b*.

Table 1. *Typical Densitometric Analysis*

DNA source	DJ_H1		DJ_H2		DJ_H3		DJ_H4		Total No./ 1 μg§	No. per organ‖
	cpm*	No.‡	cpm	No.	cpm	No.	cpm	No.		
500T	113	500	353	500	142	500	295	500	2,000	
1,000 T	202	1,000	595	1,000	195	1,000	733	1,000	4,000	
5,000 T	743	5,000	1,750	5,000	875	5,000	1,860	5,000	20,000	
+/+ BM −11¶	719	4,816	1,480	4,081	1,400	8,172	1,030	2,369	19,000	2.1×10^6
+/+ spleen-10	439	2,790	1,100	2,828	1,020	5,881	987	2,240	14,000	1.2×10^7
+/+ spleen-12	398	2,490	1,090	2,795	963	5,537	877	1,909	13,000	1.1×10^7
scid BM-17	698	4,662	273	<500 (101)	218	1,045	124	<500 (143)	6,000	6.7×10^5
scid BM-19	274	1,598	359	<500 (385)	212	1,009	862	1,864	4,900	5.4×10^5
scid BM-1	221	1,215	521	919	286	1,455	349	<500 (320)	3,900	4.3×10^5
scid spleen-18	123	506	154	<500 (209)	91.3	<500 (281)	165	<500 (266)	1,300	2.1×10^5
scid spleen-20	146	672	104	<500 (44)	41.9	<500 (483)	134	<500 (173)	1,400	2.3×10^5

See Materials and Methods for quantification.
* Output on the densitometer.
‡ Number of structures calculated using Excel TRENDS.
§ 1 μg is 1.8×10^5 cell equivalents.
‖ Calculation per organ is based on BM having 2×10^7 nucleated cells for all strains; C.B-17+/+ spleen having 1.5×10^8 nucleated cells; *scid* spleen having 3×10^7 nucleated cells.
¶ Numbers identify DNA preparations.

strains (Fig. 1, *b* and *c*, and reference 21). There were ~4.2 ± 0.5 × 10^5 DJ_H products per *scid* femur, which is ~10% that of C.B-17+/+ (3.0 ± 0.6 × 10^6). In the spleen, there were ~2.8 ± 1.0 × 10^5 DJ_H structures in *scid* and ~1.6 ± 0.3 × 10^7 in C.B-17+/+. Serum Ig^+ leaky *scid* were not significantly different from nonleaky *scid*. Although easily detectable in C.B-17+/+ BM DNA, no $DQ52J_H$ products were detected upon amplification of *scid* BM DNA using

Table 2. *Quantification of DJ_H Structure in BM and Spleen*

C.B-17 +/+ BM	*scid* BM	C.B-17 +/+ spleen	*scid* spleen
1.4×10^6	6.7×10^5	3.0×10^7	2.1×10^5
1.0×10^6*	5.4×10^5	1.6×10^7	2.3×10^5
2.1×10^6*	4.3×10^5§	1.3×10^7	5.7×10^5
6.7×10^6	2.8×10^5§	1.6×10^7‖	1.2×10^5
3.9×10^6‡	4.8×10^5§	1.1×10^7‖	
4.7×10^6‡	3.0×10^5	1.2×10^7	
1.9×10^6	3.9×10^5		
2.3×10^6			
3.3×10^6			
Average: 3.0×10^6	4.2×10^5	1.6×10^7	2.8×10^5
SE: $\pm 0.61 \times 10^6$	$\pm 0.49 \times 10^5$	$\pm 0.29 \times 10^7$	0.99×10^5

2×10^7 nucleated BM cells for all strains. 1.5×10^8 nucleated spleen cells for C.B-17+/+ and 3×10^7 cells for *scid*.
*‡§‖ The same samples evaluated in different quantifications.
Each value was determined from a set of data analyzed as detailed in Table 1.

Table 3. *Comparison of DJ_H Structures Derived from scid and C.B-17+/+ BM and BALB/c Fetal Liver (FL)*

	scid	C.B-17+/+	BALB/c FL
No. of structures analyzed	57	57	40
Average deletions (range)	8.8 (2–42)	9.1 (0–41)	6.7 (2–15)
No. of structures containing nucleotide insertions	14	47	0
Average no. of nucleotide insertions for above structures	5.4	4.2	0
RF Usage: 1	63%	44%	70%
2	7%	18%	8%
3	30%	38%	22%
Potentially productive joins	70%	61%	90%
DFL 16.1 usage	39%	81%	52%

Data are summarized from Figs. 2 and 3. BALB/c FL data are derived from Chang et al. (21).

$DQ52/J_H4$ primers. Since the sensitivity of the assay is ~40 targets/μg DNA (21), this result indicates there are $<4.4 \times 10^3$ $DQ52J_H$ rearrangements per femur (Southern analysis not shown). From these data, we conclude that *scid* BM and spleen have correctly sized DJ_H1–4 products at ~10 and ~1%, respectively, of the frequency found in C.B-17+/+.

Characterization of DJ_H Structures. Based on the lack of mature lymphoid cells in *scid* mice and the aberrant nature of the coding junctions isolated from A-MuLV-transformed lines from *scid* mice, we had expected to see few, if any, discrete normal-sized DJ_H fragments. To search for more subtle differences in the *scid* DJ_H joints, we cloned and sequenced the amplified products and compared them with those from C.B-17+/+. Fig. 2 shows the DJ_H structures derived from 6- and 27-wk-old BM of C.B-17+/+ mice. Fig. 3 shows the DJ_H structures from 6-wk-old *scid* BM. Key features of the analysis are summarized in Tables 3 and 4.

DJ_H Rearrangements in C.B-17+/+ BM. 57 DJ_H struc-

Table 4. *Comparison of DJ_H Structures with and without N or P Nucleotide Additions*

	Total *scid*		Unique *scid*		Total C.B-17+/+		Unique C.B-17+/+	
	Total	Without N or P	Total	Without N or P	Total	Without N or P	Total	Without N or P
No. of structures	57	43 (75%)	22	17 (77%)	57	9 (16%)	56	9 (16%)
RF: 1	63%	79%	55%	59%	44%	67%	45%	67%
2	7%	2%	9%	6%	18%	11%	18%	11%
3	30%	19%	36%	35%	38%	22%	38%	22%
Dfl16.1 usage	39%	21%	50%	32%	81%	90%	80%	90%
Potentially productive	70%	88%	55%	71%	61%	80%	63%	80%

Data are summarized from Figs. 2 and 3.

CLONE	DH SEQUENCE	DELETED DH SEQUENCE	INSERTIONS	DELETED JH SEQUENCE	JH SEQUENCE	DH SEGMENT	No. DELETION	No. INSERTION	RF
JH1									
B1	TT TAT TAC TAC GGT AGT	AGCTAC		CTAC	TGG TAC TTC GAT GTC TGG GGC	Df1 16.1	10	0	1
B3	TT TAT TAC TAC GGT AGT AGC	TAC	CT	CTA	C TGG TAC TTC GAT GTC TGG GGC	Df1 16.1	6	2	1
C2	TT TAT TAC TAC GGT AGT	AGCTAC	GTA TTG GTG AC	CTACTGGTA	C TTC GAT GTC TGG GGC	Df1 16.1	15	11	1
C6	TT TAT TA	CTACGGTAGTAGCTAC	T G	CTACT	GG TAC TTC GAT GTC TGG GGC	Df1 16.1	20	2	1
C1	TTT ATT	ACTACGGTAGCTAC	CGT A	CTACTGGTACTACTCG	AT GTC TGG GGC	Df1 16.1	31	0	2
C5	TTT ATT ACT ACG GTA GT	AGCTAC	C C	CTACT	GG TAC TTC GAT GTC TGG GGC	Df1 16.1	11	2	2
C4	T TTA TTA CTA CGG *TAG TAG*	CTAC	TG	CTACTGGTA	C TTC GAT GTC TGG GGC	Df1 16.1	13	2	3
B2	T TTA TTA CTA CGG *TAG TAG* C	TAC	CT C	CTACTGGTACTTCGATGTC		Df1 16.1	41	3	3
				TGGGGCACAGGGACCACGG					
C7	T TTA TTA CTA CGG *TAG TAG* C	TAC	A	CTACTG	G TAC TTC GAT GTC TGG GGC	Df1 16.1	9	1	3
C8	T TTA TTA CTA CGG TA	GTAGCTAC	C GGG	CTAC	TGG TAC TTC GAT GTC TGG GGC	Df1 16.1	12	4	3
JH2									
B6	TC TAC TAT GGT TAC GAC		GCC GGG G	ACTACTTTGACTACT	GG GGC	Dsp 2.3	15	7	1
B8	TC TAT GAT GGT TAC TAC		GAG GG	ACTACTTTGA	C TAC TGG GGC	Dsp 2.9	10	5	1
B9	TC TAT GAT GGT TAC TAC		G	ACTAC	AC TAC TTT GAC TAC TGG GGC	Dsp 2.9	0	1	1
B10	TT TAT TAC TAC GGT AGT AGC TAC		A G		TTT GAC TAC TGG GGC	Df1 16.1	5	0	1
B12	CT TAT TAC TAC GGT AGT AGC *TA*	C	A A	ACTACTTTG	AC TAC TTT GAC TAC TGG GGC	Df1 16.1	1	1	1
B13	TT TAT TAC TAC GGT AGT AG	CTAC		AC	TAC TGG GGC	Df1 16.1	13	2	1
B14	TT TAT TAC TAC GGT AGT AGC TAC			ACTAC	TTT GAC TAC TGG GGC	Df1 16.1	5	0	1
C3	TT TAT TAC TAC GGT AGT AGC	TAC	CGT TC	ACTA	C TTT GAC TAC TGG GGC	Df1 16.1	7	5	1
C9	TT TAT TAC TAC GGT AGT AGC *TA*	C	G GGG	AC	TAC TTT GAC TAC TGG GGC	Df1 16.1	3	4	1
C10	TT TAT TAC TAC GGT AGT AGC TAC		GGG	ACTAC	TTT GAC TAC TGG GGC	Df1 16.1	5	3	1
C12	TT TAT TAC TAC GGT AGT	AGCTAC	CCG CCC CC	A	C TAC TTT GAC TAC TGG GGC	Df1 16.1	11	0	1
C14	TT TAT TAC TAC GGT AGT	AGCTAC	C	ACTACTT	TT GAC TAC TGG GGC	Df1 16.1	7	9	1
C18	CC TAC TAT AGT AAC	TAC	A A	ACT	AC TTT GAC TAC TGG GGC	Dsp 2.x	9	1	1
C19	TT TAT TAC TAC GGT AGT AGC *TA*	C	A A	ACTACTTTGACTACTGGGG	C	Df1 16.1	4	2	1
B15	TTT ATT ACT ACG GTA GC	TAC	C CCA CT			Dsp 2.7 or Dsp 2.7	22	6	2
B7	TTT ATT ACT ACG GTA G	CTAC	AC GGG CAG G		AC TAC TTT GAC TAC TGG GGC	Df1 16.1	4	9	2
C13	TCT ACT ATG GTT AC	GAC		A	C TAC TTT GAC TAC TGG GGC	Dsp 2.4	4	0	2
C17	TTT ATT ACT ACG GTA G	TAGCTAC	GA GGG	ACTAC	TTT GAC TAC TGG GGC	Df1 16.1	12	5	2
B4	C CTA CTA *TAA* CTA C		AC	ACTACTTTT	GAC TAC TGG GGC	Dsp 2.x	8	2	3
B5	C CTA CTA *TG*	GT(T/A) AC(G/T) AC	A TAC TGG CGG	ACTACTTT	GAC TAC TGG GGC	Dsp 2.6	16	10	3
C16	T TTA TTA CTA CGG T	AGTAGCTAC	GC CGG ATG ATG G	ACTACT	TT GAC TAC TGG GGC	Df1 16.1	15	12	3
B11	T TTA TTA CTA CGG *TAG TAG* C	TAC	CC T	ACTA	AC TAC TTT GAC TAC TGG GGC	Df1 16.1	3	3	3
C11	T TTA TTA CTA CGG *TAG TGT* ATT CC	AGCTAC	GT ATT CC	ACTA	C TTT GAC TAC TGG GGC	Df1 16.1	10	7	3
C15	T TTA TTA CTA CGG	TAGTAGCTAC	AGG	ACTACTTT	GAC TAC TGG GGC	Df1 16.1	18	3	3
C16	T TTA TTA CTA CGG T	AGTAGCTAC	GC CGG ATG ATG G	ACTACT	TT GAC TAC TGG GGC	Df1 16.1	15	12	3
JH3									
A3	TT TAT TAC TAC GGT AG	TAGCTAC	GG		CC TGG TTT GCT TAC TGG GGC	Df1 16.1	7	2	1
A4	TT TAT TAC TAC GGT AGT AGC TAC			CC	TGG TTT GCT TAC TGG GGC	Df1 16.1	2	0	1
C36	TT TAT TAC TAC GGT AGT	AGCTAC	GAC T		CC TGG TTT GCT TAC TGG GGC	Df1 16.1	6	4	2
A1	TTT ATT ACT ACG GTA GTA G	CTAC	AG GGA AA	CCTGGTT	T GCT TAC TGG GGC	Df1 16.1	11	7	2
C23	TTT ATT ACT ACG GTA GTA GCT AC		G *TAG* G	CCT	GG TTT GCT TAC TGG GGC	Df1 16.1	3	5	2
C24	TTT ATT ACT ACG GTA GTA GCT AC		C T	CCT	GG TTT GCT TAC TGG GGC	Df1 16.1	3	2	2
A2	T TTA TTA CTA CGG *TAG TAG* CTA C			CCT	GG TTT GCT TAC TGG GGC	Df1 16.1	3	0	3
C20	T TTA TTA CTA CGG *TAG* T	AGCTAC	GG	CCTGGTTT	GCT TAC TGG GGC	Df1 16.1	14	2	3
C21,22	T TTA TTA CTA CGG *TAG* TA	GCTAC	C A		CC TGG TTT GCT TAC TGG GGC	Df1 16.1	5	2	3
C25	T TTA TTA CTA CGG *TAG TAG*	CTAC	GAG GG	C	C TGG TTT GCT TAC TGG GGC	Df1 16.1	5	5	3
C26	T TTA TTA CTA CGG *TAG TAG* CTA	C	TGG GAC TTT	CCTGG	TTT GCT TAC TGG GGC	Df1 16.1	6	9	3
C27	C CTA CTA TAT GCC	G(G/T) (T/A) AC(G/T) AC	T GCC	CCTGG	TTT GCT TAC TGG GGC	Df1 16.1 or Dsp 2.x	13	4	3
C28	T TTA TTA CTA CGG *TAG TAG* CC	TAC		CCTG	G TTT GCT TAC TGG GGC	Df1 16.1	7	0	3
C35	T TTA TTA CTA CGG *TAG TAG* CTA C		GC	CCTGGTTT	GCT TAC TGG GGC	Df1 16.1	8	2	3
JH4									
A6	TT TAT TAC TAC GGT AGT AGC TAC		GTG AGG	ATTACTAT	GCT ATG GAC TAC TGG	Df1 16.1	8	6	1
C29	TT TAT TAC TAC GGT AGT AGC TA	C		A	T TAC TAc GCT ATG GAC TAC TGG	Df1 16.1	2	0	1
C30	CC TAC TAT AGT AA	CTAC	G G	ATTACT	AT GCT ATG GAC TAC TGG	Dsp 2.x	10	2	1
C31	TT TAT TAC TAC GGT AGT AG	CTAC	G GGG G	ATTACT	AT GCT ATG GAC TAC TGG	Df1 16.1	10	5	1
C33	TT TAT TAC TAC GGT AGT AGC T	AC	GC T		AT TAC TAT GCT ATG GAC TAC TGG	Df1 16.1	2	3	1
A5	T TTA TTA CTA CGG *TAG* TA	GCTAC	C	AT	TAC TAT GCT ATG GAC TAC TGG	Df1 16.1	7	1	3
C32	T CTA *TGA* TGG TTA CTA	C	T		AT TAC TAT GCT ATG GAC TAC TGG	Dsp 2.9	1	1	3
C34	C CTA CTA *TAG TAA* C	TAC	CA CGG ATC CT	ATTA	C TAT GCT ATG GAC TAC TGG	Dsp 2.x	7	10	3

Figure 2. DNA sequences of C.B-17+/+ DJ_H structures analyzed. Clones designated by A and B represent separate amplifications of BM DNA from a single 6-wk-old C.B-17+/+ mouse. Clones designated with a C are derived from a 27-wk-old C.B-17+/+ mouse. The nucleotides deleted from or added to the germline sequence of the recombining elements are indicated. In some structures the deleted nucleotides could have come from either the D_H or J_H element. Possible P insertions are underlined. In-frame stop codons are bold and italicized. Possible point mutations or Taq-induced errors are in lower-case letters.

CLONE	DH SEQUENCE	DELETED DH SEQUENCE	INSERTIONS	DELETED JH SEQUENCE	JH SEQUENCE	DH SEGMENT	No. DELETION	No. INSERTION	RF
JH1									
Aii 1,2	TC TAT GAT GGT TAC TAC			CTAC	TGG TAC TTC GAT GTC TGG GGC	Dsp 2.9	4	0	1
Di 1-3	TTT ATT ACT ACG GTA GTA G	CTAC	TT	CTAC	TGG TAC TTC GAT GTC TGG GGC	Dfl 16.1	8	2	2
JH2									
Ai 2	TC TAT GAT GGT TAC T	AC		ACTACTTTGTCTACT	GG GGC CAA GGC	Dsp 2.9	17	0	1
Ai 3*	TC TAC TAT G	GTAACTAC		ACT (JH2)	AC TTT GAC TAC TGG GGC CAA GG	Dsp 2.1-2.5	42	0	1
				ATTACTATGCTATGGACTACT GGGGTCAAGG (JH4)	A ACC TCA GTC				
Ai 1	T TTA TTA CTA CGG ***TAG TAG*** CT	AC		ACT	A CTT ***TGa*** CTA CTG GGC CAA GGC	Dfl 16.1	5	0	3
Bi 2	C CTA CTA ***TAG*** T	AACTAC		ACTACT	TT GAC TAC TGG GGC CAA GGC	Dsp 2.x	12	0	3
Ai 4	C CTA CTA ***TAG TAA*** CTA C		GT AGG ATT	AC	TAC TTT GAC TAC TGG GGC CAA GGC	Dsp 2.x	2	8	3
Bi 3	T TTA TTA CTA CGG TA	GTAGCTAC		ACTACTTTGACTA	C TGG GGC CAA GGC	Dfl 16.1	21	0	3
JH3									
Aii 3	T CTA CTA ***TGA*** TTA CGA C			CCTGGTTTG	CT TAC TGG GGC	Dsp 2.2	9	0	3
Ci 1,Cii 1 Ciii 1,2 Civ 1-4	T TTA TTA CTA CGG ***TAG TAG*** CTA C		CC CCG TT	CCTGGTTTGC	T TAC TGG GGC	Dfl 16.1	10	7	3
JH4									
Ai 5	TT TAT TAC TAC GGT AGT AGC ***TA***	C	A T		AT TAC TAT GCT ATG GAC TAC TGG GGT	Dfl 16.1	1	2	1
Aii 4	TT TAT TAC TAC GGT AGT AGC TA	C		ATTACTA	T GCT ATG GAC TAC TGG GGT	Dfl 16.1	8	0	1
Aii 9,10	TT CAT TAC TAC GGC TA	C		ATTACTA	T GCT ATG GAC TAC TGG GGT	Dfl 16.2	8	0	1
Cii 2	TT TAT TAC TAC GGT AGT AGC TAC			ATTAC	TAT GCT ATG GAC TAC TGG GGT	Dfl 16.1	5	0	1
Ciii 3	TT TAT TAC TAC GGT AGT AGC T	AC	GC T		AT TAC TAT GCT ATG GAC TAC TGG GGT	Dfl 16.1	2	3	1
Ciii 4	TT TAT TAC TAC GGC GGT AGT AGC TA	C		A	T TAC TAT GCT ATG GAC TAC TGG GGT	Dfl 16.1	2	0	1
Ciii 5	TT cAT TAC TAC GGT AGT AGC T	AC			AT TAC TAT GCT ATG GAC TAC TGG GGT	Dfl 16.1	2	0	1
Ciii 6	TC TAC TAT GAT TAC GAC			ATTAC	TAT GCT ATG aAC TAC TGG GGT	Dsp 2.2	5	0	1
Ci 2-8,9 Ciii 8-21 Civ 5	TT CAT TAC TAC GG	CTAC		ATTA	C TAT GCT ATG GAC TAC TGG GGT	Dfl 16.2	8	0	1
Bi 1	CCT AGT ATG GTA AA	CTAC		ATTACTATG	CT ATG GAC TAC TGG GGT	Dsp 2.8	13	0	2
Aii 5-7	T TTA TTA CTA CGG ***TAG*** TA	GCTAC		ATTACTA	T GCT ATG GAC TAC TGG GGT	Dfl 16.1	12	0	3
Aii 8	C CTA CTA ***TAG*** GT	ACGAC		ATTA	C TAT GCT ATG GAC TAC TGG GGT	Dsp 2.11	9	0	3

Figure 3. DNA sequences of the nonleaky 6-wk-old *scid* BM DJ_H structures. A, B, and C designate different *scid* mice. Lower-case Roman numerals represent separate PCR primary and secondary amplifications. The nucleotides deleted from or added to the germline sequences of the recombining elements are indicated. In some cases deleted nucleotides may have come from either recombining element. Possible P insertions are underlined. In-frame stop codons are italicized and bold. * A DJ_H structure with a fusion of a partial J_H2 to J_H4. Mutations from germline sequences are indicated by lower-case letters. Clones whose mutations are not shown in this chart have their clone name bold.

tures from C.B-17+/+ were analyzed. Since no difference was observed between structures derived from 6- and 27-wk-old mice, the data in Fig. 2 are pooled from both sources. Deletions were present in most coding joints. An average of 9.1 nucleotides was deleted from D and/or the J_H. N and P insertions, which are a common feature in adult Ig gene rearrangement (26), were observed in 82% of the structures, with an average of 4.2 nucleotides being added. Nine structures contain possible P nucleotides, of which seven also have N insertions.

Although D_H elements can be read in all three reading frames (RF) and in either orientation, RF usage is not random. In the C.B-17+/+ BM, RFs 1, 2, and 3 were used in 44, 18, and 38% of the structures, respectively. RF1 is most markedly overused in the fetal repertoire where there are few if any N additions due to the lack of terminal deoxynucleotidyl transferase (TdT) activity (27). Terminal homologies of the recombining elements are thought to promote the RF1 usage in cases where there is no N addition (21, 26, 28). Indeed, upon analysis of structures within N nucleotides (nine structures), the RF1 bias becomes apparent: 67:11:22% (Table 4).

Of the 57 C.B-17+/+ structures, 35 (61%) were potentially productive; i.e., they lack stop codons in the used reading frame and invariant residues are present in the J_H element. DJ_H3 structures were the least productive (40%) due to the more frequent usage of RF3, which has many stop codons. The biased D usage reported by us (21) and others (29, 30) was also present; genetic element Dfl16.1 was used in 81% of the DJ_H rearrangements.

DJ_H Rearrangements in scid BM. DNA derived from the BM of three 6-wk-old, Ig^- *scid* mice was individually amplified and cloned. Fig. 3 shows the sequences of 57 DJ_H structures from *scid* BM DNA, and key features are summarized in Tables 3 and 4.

The *scid* J_H structures shown in Fig. 3 are, in fact, quite similar to those of C.B-17+/+ mice (Fig. 2) and other strains (21, 26, 28). However, there are marked quantitative and qualitative differences, particularly in the degree of diversity. Of 57 *scid* structures isolated, only 22 were unique; 56 of 57 C.B-17+/+ DJ_H were unique. Moreover, only 14 of the 57 *scid* structures (5 of the 22 unique ones) contained N or P nucleotide insertions. Of the five unique structures containing N or P, two contained N only, two contained possible P and N, and one structure contained possible P only. The average number of insertions for structures containing N and/or P was 5.4 (4.4 for unique structures), somewhat more than C.B-17+/+ (4.2 nucleotides [nt]). The mean number of nucleotides deleted from the recombined coding ends was 8.8, which is about the same as C.B-17+/+ (9.1 nt) (Table 3).

scid DJ_H structures used RF1 more frequently than C.B-17+/+. Of the 57 *scid* DJ_H structures, 63, 7, and 30% used RFs 1, 2, and 3 respectively (compared with 44, 18, and 38%, respectively, for C.B-17+/+). Interestingly, RF1 was used more frequently in both strains when structures joined without N and/or P insertions (Table 4). 70% (55% of the 22 unique structures) of the *scid* structures could yield a functional Ig protein as defined by the lack of stop codons and the presence of invariant residues in the J_H element. None of the *scid* DJ_H3 structures were potentially productive. Dfl16.1 was overused, but less frequently than in C.B-17+/+. 39% of the *scid* (50% if the unique only are considered) structures use this D_H element (Table 4).

Discussion

Functional DJ_H Joints in scid Lymphoid Progenitors. In A-MuLV-transformed lymphocytes from *scid* mice, all DJ_H coding joints were grossly abnormal, containing large deletions of both D and J_H regions (5, 9). Therefore, we expected to observe few, if any, DJ_H joints in cells from *scid* bone marrow. To our surprise, normal DJ_H joints were observed in the present study. Moreover, the frequency of progenitor cells containing potentially functional joints was also close to normal. We arrived at this latter conclusion by determining the frequency of normal DJ_H joints detected by PCR and the proportion of cells in BM belonging to the B cell lineage. In normal BM, ~30% of the cells belong to the B lineage. Of these cells, approximately two-thirds are in the pre-B cell stage (i.e., contain cytoplasmic μ) or B cell stage (i.e., express surface Ig). The remaining cells are in the pro-B stages. According to a recent study by Osmond et al. (31), the early and intermediate pro-B cells in *scid* mice are normal in frequency and proliferation kinetics. The late pre-B stage is markedly depleted in *scid* mice, so that overall the number of B lineage cells in the BM of *scid* mice is only ~10% of the number found in the BM of normal mice. Since *scid* mice contain ~10% as many DJ_H joints as normal mice (Table 2), many of the pro-B cells in *scid* mice must contain potentially functional DJ_H joints.

These data appear to contradict previously published data on the frequency of normal DJ_H joints in pro-B cells transformed by A-MuLV (5, 9, 32) and those pro-B cells produced in long-term BM cultures (10, 33) derived from *scid* mice. In both instances, few, if any, normal DJ_H joints were observed in cells from *scid* mice. Several factors may contribute to this discrepancy: The low number of $B220^+$ cells in the BM of *scid* mice (31) indicates that cells that fail to make a functional heavy chain gene are rapidly deleted in the BM. However, cells with nonfunctional rearrangements may be rescued either by transformation with A-MuLV or by the growth conditions in long-term BM cultures. It is also possible that during the continued growth of these rescued cells the abnormal recombinase system continues to function so that further gross deletions are generated under these two conditions. Thus, the abnormal rearrangements may be a result of the rescue of cells from programmed cell death, and they may not accurately reflect the ability of the recombinase system to carry out DJ_H recombination.

It is also possible that the major defect in gene rearrangement occurs when the cells attempt to form a V_H-DJ_H joint. Quasi-normal DJ rearrangements have been reported in *scid* thymocytes at the δ locus, while there was no evidence of Vδ rearrangement (20). Studies in long-term BM cultures

and in A-MuLV-transformed cells provided little evidence for attempted V to DJ recombination, though it is possible that activation of this process is highly abnormal and leads to the gross deletions mentioned previously. Using primers that detect a large proportion of the V_H joints, we are attempting to investigate VDJ_H recombination with similar quantitative PCR studies of the type described in this paper.

Implications for the Leaky Phenotype. As mentioned above, *scid* is a leaky phenotype; normal Ig-secreting lymphocytes arise in *scid* mice (12). One model put forward to explain leakiness is the somatic reversion model. The premise of this model is that a genetic reversion in a pro-B or earlier cell leads to clones of cells capable of normal gene rearrangement. The major piece of evidence supporting the somatic reversion model is the observation of normal coding joints on the nonexpressed alleles in T cells obtained from leaky *scid* mice (16). This observation was taken as evidence that the recombination machinery was normal in these cells and hence that a reversion had taken place. Our hypothesis is, instead, that leakiness is the result of the chance occurrence of three functional Ig rearrangements in a single lymphoid cell. Indeed, if a cell ultimately makes a functional receptor (the requirement for leakiness), it is not unlikely that the rearrangement on the other allele is also functional. This explanation would account for the data from Petrini et al. (16), as described above, and does not require that there was a reversion in the recombinase machinery itself.

Fetal Nature of DJ_H Joints in scid Mice. Although the proportion of potentially functional DJ_H joints in *scid* mice was similar to that observed for normal mice, there were unusual features of the joints in *scid* mice. Very few additional (N or P) nucleotides are inserted into the joint. A similar low frequency is often observed in fetal liver cells (21, 26, 28) and is attributed to the low levels of TdT in fetal liver (27, 28). As reported by Osmond et al. (31), the frequency of TdT^+ cells in *scid* BM is identical to that observed in the BM of normal mice. Thus, the low level of N nucleotides is unlikely to result from overall reduced TdT levels.

The second unusual feature of the DJ_H rearrangements in *scid* mice was the restricted repertoire observed. When we sequenced 57 DJ_H joints isolated from C.B-17+/+ mice, we detected 56 unique sequences. In contrast, the 57 DJ_H clones from *scid* mice contained only 22 unique sequences. Six of these sequences were isolated many times. If, as discussed above, the *scid* mutation results in a dissociation between the timing of gene rearrangement and the sequential transition of cells from pro-B to pre-B to B cells, it is possible that an abnormal expansion occurs in the late pro-B cell stage allowing minor clonal dominance of some DJ_H rearrangements. Support for this explanation can be seen in some of the Southern blots (Fig. 1, *b* and *c*) where, at times, one of the four DJ_H bands is unusually intense. Such band may represent an expanded clone.

Recently, possible intermediates in V(D)J recombination have been detected in *scid* thymocyte DNA. These intermediates are "hairpinned" Dδ and Jδ coding ends. The inability to isolate such structures from normal mouse DNA has lead to the speculation that the *scid* defect affects the resolution and/or generation of these structures (2, 34). Taken with our finding of a general lack of N insertions in *scid*, these data suggest that the *scid* product might interact, or rather interfere, with TdT activity at the stage where the hairpins are resolved. In view of the observations that homologies at the coding ends promote recombination in the absence of TdT (21, 26), examination of DJ_H joints in *scid* fetal liver, where recombination occurs solely without N addition, may be informative.

We are thankful for the careful assistance of Clare Ellen Spencer and Lynne Omoto. We thank Dale Ramsden, Michael Atkinson, and Chris Paige for helpful discussions.

This work was funded by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada with funds from the Terry Fox Marathon of Hope. G. Wu is an Medical Research Council Scholar.

Address correspondence to Gillian E. Wu, Wellesley Hospital Research Institute, 160 Wellesley Street, East Toronto, Ontario, Canada M4Y 1J3.

Received for publication 4 May 1993 and in revised form 21 June 1993.

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