The *scid* gene encodes a *trans*-acting factor that mediates the rejoining event of Ig gene rearrangement

Eric A. Hendrickson,^{1,2} David G. Schatz,^{3,4} and David T. Weaver^{1,2,5}

¹Division of Tumor Immunology, Dana-Farber Cancer Institute, and ²Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115 USA; ³Whitehead Institute for Biomedical Research, and ⁴Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 USA

Homozygous mutation at the *scid* locus in the mouse impairs lymphoid development and results in animals deficient in B and T cells. We found that immunoglobulin heavy-chain gene rearrangement was blocked at the *D*–*J_H* stage in Abelson-transformed *scid* pre-B cell lines. Examination of the recombinational junctions indicated that the correct gene elements (*D* and *J_H*) were assembled, as shown by the presence of *D* region and *J_H*-region DNA on the breakpoint restriction fragments cloned from the genome of the *scid* cell lines. All rearrangement events were accompanied by deletions of varying sizes such that none of the rearrangements resulted in the production of functional immunoglobulins. The breakpoints of the rearrangement events did not correspond to the utilization of a novel heptamer–nonamer recombination signal but probably arose by nonspecific deletion from distal *J_H* and *D* heptamer–nonamer signals in the process of recombination. *scid* pre-B cell lines were infected with a recombinant retrovirus (DGR) containing Ig joining signals. Aberrant rearrangements were observed in DGR DNA that was integrated randomly throughout the mouse genome, which suggested that the mutation in *scid* mice encodes a *trans*-acting factor that is part of the lymphoid gene recombination machinery.

[Key Words: *scid*; immunodeficiency; immunoglobulin gene rearrangements; lymphoid development]

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DNA rearrangement of lymphoid genes is restricted in differentiation to the early stages of B- and T-cell maturation. The recombination events that join variable region gene segments (*V*, *D*, and *J*) of any of the immunoglobulin (Ig) or T-cell receptor (TCR) gene families occur in distinct tissue-specific and temporal patterns (Alt et al. 1986; Kronenberg et al. 1986). In the mouse, *D*–*J_H* joining always precedes *V*–*DJ_H* joining in Ig heavy-chain gene rearrangement (Alt et al. 1984). Two lines of evidence suggest that an enzymatic activity [*V(D)J* recombinase] mediates all the lymphoid gene rearrangement events: (1) Conserved *cis*-acting heptamer–nonamer DNA sequences flank the variable region gene segments of all the gene segment families (Early et al. 1980; Sakano et al. 1981; Tonegawa 1983; Davis 1985), and (2) exogenous TCR or Ig recombination signals can stimulate DNA rearrangement when introduced into pre-B cell lines (Blackwell and Alt 1984; Lewis et al. 1984, 1985; Yancopoulos et al. 1986). Other proteins or factors are presumed to modulate the activity of the *V(D)J* recombinase at subsequent rearrangement steps. With the exception of terminal deoxynucleotidyl transferase (TdT), implicated in *N*-region formation (Alt and

Baltimore 1982; Desiderio et al. 1984; Landau et al. 1987), none of the enzyme activities responsible for Ig and TCR gene recombination have been identified.

Homozygous mutation of the *scid* locus in the mouse disrupts both B- and T-cell lymphoid development, resulting in immeasurable or low serum immunoglobulin levels, the absence of cells bearing T- or B-cell determinants, and lack of functional T cells as assayed by skin allograft rejection (Bosma et al. 1983). The *scid* defect specifically affects the lymphoid subset of the hematopoietic lineages because myeloid and erythroid differentiation are normal (Dorschkind et al. 1984). Aberrant rearrangement of Ig and TCR genes have been found in *scid* Abelson murine leukemia virus (Ab-MLV)-transformed bone marrow cells and spontaneous thymic lymphomas (Schuler et al. 1986). In these cells, Ig heavy-chain gene rearrangements and TCR β -gene rearrangements were accompanied by a high incidence of deletions that removed all or part of the *J_H* region and the *J* β 2 locus, respectively. These data suggested that the *scid* mutation altered some component of the recombination system common to both lineages (Schuler et al. 1986).

We would like to understand where the *scid* mutation acts in lymphoid differentiation and what process in the

⁵Corresponding author.

DNA rearrangement of lymphoid genes is disrupted. In this paper we show that the first rearrangement events of pre-B cells (D_H – J_H) are defective in *scid* mice. Cloned restriction fragments from the J_H region demonstrated that the breakpoint junctions were generated by joining D -element regions to the J_H region, as is observed for wild-type cells. However, although the appropriate J_H and D_H chromosomal regions were selected, no *scid* D_H – J_H rearrangement was compatible with the synthesis of an Ig gene product. The precise junctions of nine independent breakpoints were characterized: None flanked consensus DNA sequences that resembled, in any way, the conventional heptamer–nonamer recombination signals. The breakpoint sequences appear to have arisen by nonspecific deletions originating from the J_H and D_H signal sequences during the process of recombination. In *scid* cell lines with an integrated copy of a recombinant retrovirus containing Ig rearrangement heptamer–nonamer sequences, subsequent rearrangements of these sequences also produced nonspecific deletions. These data suggest that the *scid* gene encodes a *trans*-acting factor involved in lymphoid gene rearrangement that acts at the joining stage of the recombination mechanism.

Results

scid pre-B cell lines have faulty Ig heavy-chain rearrangements

Pre-B cells from the bone marrow of a *scid* homozygous mouse were transformed with Ab-MLV (Rosenberg and Baltimore 1976; Weaver et al. 1985), and 12 independent pre-B-cell lines were established. Genomic DNA isolated from these cell lines was digested with *Eco*RI and analyzed by Southern blots to detect DNA rearrangements in the J_H region by using a hybridization probe from the 3'-side of the J_H region (Fig. 1). In wild-type pre-B-cell lines transformed by Ab-MLV, both alleles normally have undergone Ig heavy-chain D/J_H or $V_H D/J_H$ rearrangements, resulting in new *Eco*RI sites flanking the J_H region (see Fig. 2) and thus generating two new hybridizing bands. When we tested the *scid* cell line DNAs by this protocol, the Ig rearrangement state of the two alleles fell into three classes: (1) no hybridizing bands: 3C; (2) only one hybridizing band: 7B, 2B, 3D, 8E, 8B, 8D, and 6A; and (3) two hybridizing bands: 2A, A, A2, and 7A (Fig. 1). By comparison to unrearranged non-lymphoid DNA from wild-type mice (BL6, Fig. 1), all the hybridizing bands in the *scid* cell lines were from rearranged alleles. Cell lines with either one or no hybridizing bands presumably deleted the region complementary to the 3'-probe on one or both alleles, respectively. The *scid* cell lines with two hybridizing bands may have undergone wild-type rearrangement events or may have undergone aberrant events in which the region homologous to the probe simply was retained. These data supported an interpretation similar to that of Schuler et al. (1986), who postulated that the *scid* mutation disrupted normal Ig gene rearrangement. We examined rearrange-

ment of the Ig κ light-chain genes by Southern blot analysis with a J_κ hybridization probe (Weaver et al. 1985), but no indication of loss of alleles or κ rearrangements in any of the *scid* cell lines was found (data not shown). Most wild-type Ab-MLV cell lines lack light-chain rearrangements, suggesting that they are transformed during the process of Ig heavy-chain rearrangement. Thus, the rearrangement status of the *scid* cell lines indicated that they were transformed at a time in differentiation similar to that of wild-type pre-B cells.

We determined the nature of the rearrangement defect in the *scid* cell lines by complete characterization of several of the rearrangement events. The *Eco*RI fragments containing the Ig heavy-chain rearrangement breakpoints from nine independent *scid* rearrangements were cloned, utilizing the same hybridization probe (3'-probe, Figs. 1 and 2) and size-selected phage λ gt10 libraries from the *scid* cell lines (see Experimental procedures). The *Eco*RI breakpoint fragments from the J_H cluster sub-

Figure 1. DNA rearrangement in the J_H region in *scid* pre-B cell lines. (Top) Genomic DNA was isolated from 10 independently-derived Ab-MLV *scid* cell lines (7B–6A), digested with *Eco*RI, and fractionated on an 0.8% agarose gel for Southern blot analysis. C57BL/6 liver DNA (BL6) shows the 6.4-kb unrearranged *Eco*RI fragment of the J_H region. A 0.7-kb *Xba*I–*Eco*RI fragment (3' probe) was used as the hybridization probe. (Bottom) The 6.4-kb germ-line *Eco*RI fragment containing the Ig J_H region. (Arrows), *Eco*RI sites; (triangles), heptamer–nonamer recombination signals flanking the D and J_H gene segments (open rectangles).

Figure 2. *scid* rearrangements in the J_H region are defective. (Top) Wild-type $D-J_H$ rearrangements occur by the joining of a D element from the D region to one of the four J_H elements. These rearrangements are characterized by the generation of novel *Eco*RI fragments hybridizing to the 3' probe and breakpoints that occur at the junction of the D and J elements mediated by the heptamer-nonamer recombination sequences (triangles). (Bottom) The *Eco*RI fragments cloned from the J_H region of nine independent rearrangements in *scid* pre-B cell lines. Designations in the right column refer to the *scid* Ab-MLV cell line of Fig. 1 from which the breakpoint fragment originated. The numbers following the cell line designation only refer to individual isolates derived from the λ phage DNA libraries for each *scid* cell line. The thick horizontal line represents DNA from the D region, with the exception of the D_{Q52} element. (*) The breakpoint junction.

sequently were subcloned into pUC19 (Fig. 2). Restriction enzyme analysis showed that each of the clones differed from the germ-line 6.4-kb *Eco*RI fragment in their 5'-regions (data not shown). The point at which the *scid* J_H -region DNA diverged from the known J -region sequence was localized to within 100 bp, using fine-scale restriction mapping. The DNA sequences of the nine breakpoint-containing regions were determined [Experimental procedures]. In normal Ig rearrangement, 1 of ~12

D_H elements, located over 20–90 kb 5' to the J region (Kurosawa and Tonegawa 1982), is joined to one of the 4 J_H elements, placing the breakpoint at the 5'-border of one of the four J_H elements (wild type, Fig. 2). In all cases of the *scid* rearrangements, the location of the breakpoints demonstrated that each joining event was defective (*scid*, Fig. 2). Even in two cases of *scid* cell lines where two rearranged J_H alleles were observed with the 3'-probe (7A and 2A in Fig. 1), the rearrangements char-

acterized for both alleles were abnormal (7A1-2 and 7A2-2; 2A3-3 and 2A5-3). Thus, *scid* pre-B cell lines could not generate precise functional D/J_H or VD/J_H rearrangement events.

Aberrant *scid* $D-J_H$ rearrangements are produced

We tested whether the *scid* rearrangements that occurred in the J_H cluster were the result of attempted D_H-J_H joining, which ordinarily occurs at the pre-B cell stage by probing the *scid* breakpoint clones for D-element homology using the relatively conserved 5'-flanking DNA sequences of Ig D_H elements (Kurosawa and Tonegawa 1982; Alt et al. 1984). Sequences in the immediate 5'-flanking region of the D_{FL16} , D_{SP2} , and D_{Q52} families hybridize to other members of the same family and exhibit substantial cross-hybridization to members of the other two families. A *SacI*-*Bgl*III fragment ('D' probe), located ~500 bp from the 5'-end of the $D_{SP2.7}$ element isolated from p40E4-2-5.2 (Desiderio et al. 1984), was used as a hybridization probe for a Southern blot of

the *scid* *Eco*RI breakpoint restriction fragments (Fig. 3). As controls, the parent plasmid (p40E4-2-5.2) and a plasmid containing the 5'-flanking sequences of a D_{FL16} element (p38B9; Alt and Baltimore 1982) were included. As expected, the D probe hybridized to the parent plasmid from which it was derived and cross-hybridized to the D_{FL16} plasmid (Fig. 3A, lanes 1 and 2). In addition, two of the *scid* clones, A3-7 and 7A1-2, showed strong homology to the D probe (lanes 3 and 6). An identical Southern blot probed with the 5'-flanking sequence of the $D_{FL16.1}$ element from p38B9 hybridized only to the same two *scid* clones (data not shown). We localized the region of homology to the D probe in clone 7A1-2 to a 1.3-kb *Pst*I fragment by additional Southern blotting (Fig. 3B, top). However, because the D probe homology was located only ~200 bp away from the breakpoint, we presumed that the flanking D element had been deleted during rearrangement. In clone A3-7, the region of D homology was also localized near the breakpoint. A previously uncharacterized D element (named $D_{SP2.10}$) was identified only 51 bp, 5' of the breakpoint, by DNA se-

Figure 3. DNA rearranged into the J_H cluster contains D elements. (A) Southern blot of the *scid* breakpoint-containing plasmids digested with *Eco*RI and probed with D-region DNA sequences. The hybridization probe (p40E4) was the 0.5-kb *SacI*-*Bgl*III fragment from the 5'-flanking region of a D element in the plasmid p40E4-2-5.2. p38B9 and p40E4 are plasmids containing D/J_H rearrangements and D_{FL16} and D_{SP2} elements, respectively (Alt and Baltimore 1982; Desiderio et al. 1984). (B) The 6.8-kb *Eco*RI fragment, 7A1-2, that hybridized to the D probe is shown. By an additional Southern blot, the D probe homology was localized to the 1.3-kb *Pst*I fragment spanning the breakpoint. This homology region was split by digestion with *Hinc*II, located 400 bp upstream of the breakpoint (data not shown). Because the region of homology for the D probe was so close to the breakpoint, the flanking D element for 7A1-2 was presumably within 200 bp of the breakpoint position. The 4.0-kb *Eco*RI fragment, A3-7, that hybridized to the D probe is shown. By DNA sequencing, a previously uncharacterized D element was localized 51 bp from the *scid* breakpoint. The nonamer and heptamer sequences are boxed flanking the $D_{SP2.10}$ element. (*) The position of the breakpoint. (S) *SacI*; (Bg) *Bgl*III; (P) *Pst*I; (H) *Hinc*II; (arrows), *Eco*RI.

quencing (Fig. 3B, bottom). $D_{SP2.10}$ appears to be a member of the D_{SP2} family, differing by only 2 or 3 nucleotides from other family members (Kurosawa and Tonegawa 1982). $D_{SP2.10}$ contains the canonical heptamer–nonamer sequences necessary for recombination and, by this criteria, is fully functional for rearrangement; however, $D_{SP2.10}$ was probably not the element used in the rearrangement event that produced clone A3-7, because its 3' heptamer–nonamer sequences were completely intact. It is more likely that a D element 3' to $D_{SP2.10}$ was used in the rearrangement but was then subsequently deleted such that the juxtaposition of $D_{SP2.10}$ with the breakpoint was coincidental. These data strongly suggested that DNA from the D_H region was used in the recombination events that generated clones 7A1-2 and A3-7. The other *scid* cell lines may also contain DNA from the D_H region in the breakpoint fragments but may have deleted the DNA sequences that cross-hybridize to the D probe.

To address this question further, we utilized a second assay to identify the chromosomal origin of the novel DNA sequences in the 5'-flanking region of the breakpoint-containing fragments. Genomic DNAs were prepared from mouse liver DNA and BAF3-12 cell lines (germ-line Ig configuration), four wild-type Ab-MLV pre-B cell lines containing normal D/J_H rearrangements on both alleles (DJ), and five wild-type Ab-MLV pre-B cell lines containing normal VD/J_H rearrangements on both alleles (VDJ , Fig. 4A), where the Ig rearrangement status of these lines had been documented previously (Alt et al. 1984). The VDJ_H rearranged cell lines have deleted the entire D_H region (except for the 10–20 bp that constitute the D element) as a consequence of normal heavy-chain rearrangement. The DJ_H rearranged cell lines have deleted some, all, or none of the D_H region DNA, depending on the position of the D elements participating in DNA rearrangement. Hybridization probes were prepared from small restriction fragments of four

Figure 4. DNA rearranged into the J_H cluster originates from the D region. (A) Determination of the chromosomal region of origin of DNA joined to the J_H region in *scid* rearrangements. Ig heavy-chain gene rearrangement proceeds in two stages: the joining of a D to a J element followed by the joining of a V to a DJ element. Ab-MLV pre-B cell lines have been isolated with both alleles at DJ , or with both alleles at VDJ , as well as other combinations. We have used a set of normal Ab-MLV cell lines with known Ig rearrangement status to specify the origin of the joined DNAs. (See text for a further explanation of the assay.) (B) Southern blot analysis of wild-type Ab-MLV pre-B cell lines probed with *scid* breakpoint fragment hybridization probes. The four *scid* clones used (shown on the far right) are 3D26A-3, 1.0-kb *Bam*HI (B) fragment; 8B-8, 150-nucleotide *Hind*III (H)-*Sca*I (S) fragment; 7B5-3, 500-nucleotide *Hind*III (H) fragment; and 2B5-3 500-nucleotide *Bgl*II (Bg)-*Hinc*II (Hc) fragment. The DNA used in these blots was derived from cell lines described in Experimental procedures and the text and consisted of five VDJ lines, four DJ lines, and two germ-line samples.

scid clones, each from the 5'-region of the breakpoint fragment (Fig. 4B), and used to probe Southern blots containing *Eco*RI-digested genomic DNA from the set of unrearranged, partially, or completely rearranged wild-type cell lines above. If the normal genomic position of the probe is in the D_H region, this position will be deleted from all cell lines with VDJ_H rearrangements and some of the cell lines with DJ_H rearrangements. Hybridization probes from *scid* clones 3D26A-3 or 8B-8 hybridized only to DNA from unrearranged cell lines but not to any of the DJ_H or VDJ_H rearranged cell lines (Fig. 4B, 3D26A-3 and 8B-8). Hybridization probes from clones 7B5-3 or 2B5-3 hybridized to a series of DNA fragments in the DJ_H and the germ-line configuration cell lines but did not hybridize to any of the VDJ_H rearranged cell lines (Fig. 4B, 7B5-3 and 2B5-3). The multiple bands detected by the 7B5-3 and 2B5-3 probes presumably resulted from cross-hybridization of the probe with the flanking sequences of other *D* elements. Three other probes derived from the *scid* breakpoint fragments of clones A3-7, 2A5-3, and 2A3-3 showed hybridization to germ-line and DJ_H but not VDJ_H rearranged cell lines (data not shown). These data strongly argue that the rearranged DNA in all of the *scid* clones originated from positions in the D_H region. Thus, in the six *scid* rearrangements examined in this fashion, legitimate $D-J_H$ joining events were attempted, although the breakpoint junctions derived were aberrant.

Recognition of heptamer–nonamer recombination sequences in *scid* rearrangements

Ig and TCR gene rearrangements are promoted by conserved heptamer and nonamer sequences flanking the Ig and TCR gene segments. In Ig $D-J_H$ recombination, the *D* element is joined to the *J* element with deletion of the intervening DNA, which contains the heptamer–nonamer signal sequences at its 5' and 3' termini (wild-type positions I and II, Fig. 5A). To identify the signal sequences used in recombination, one can determine the breakpoint in the rearranged DNA and then extrapolate back to the flanking DNA sequences of the germ-line configuration (Fig. 5A). The same logic was used to determine whether the aberrant *scid* rearrangements resulted from the recognition of altered heptamer–nonamer signals located at other positions in the *D* and J_H regions by the *scid* recombinational machinery. The DNA sequences flanking the *scid* breakpoints that were removed by deletional rearrangements in the J_H region were identified by DNA sequencing across the recombination junctions and comparing these sequences to the known J_H region DNA sequence (position I, Fig. 5B). There was no correlation between the DNA sequences found flanking the *scid* breakpoints and heptamer–nonamer sequences found bordering normal rearrangement breakpoints. Similarly, there was no common heptamer-like or heptamer–nonamer-like DNA sequences or any other consensus DNA sequence among the nine *scid* rearrangements, either at the breakpoint junctions or within 100 bp 5' to the breakpoint in the J_H region exam-

ined (data not shown). Thus, the *scid* defect probably does not cause aberrant rearrangements by allowing the recombinational machinery to recognize altered or variant heptamer–nonamer sequences in the J_H region.

To determine which DNA sequences were deleted from the flanking area of the breakpoint junctions in the *D* region (position II, Fig. 5A), we cloned the genomic DNA from the *D*-region side of the breakpoint fragments for two *scid* clones (3D26A-3 and 7B5-3) from a wild-type genomic DNA λ phage library (see Experimental procedures). *Eco*RI fragments derived from the positive phage clones were subcloned into pUC19 (614-4 and 709T-1, see Fig. 6). By comparing the restriction digestion pattern of 3D26A-3 with *scid* clone 8B-8, it was apparent that the *D*-region sequences in 3D26A-3 and 8B-8 were from the same *Eco*RI restriction fragment, although they contained independent breakpoints. Thus, clone 614-4 allowed the determination of the *D*-region composition both for clones 3D26A-3, from which 614-4 was derived, and for 8B-8. We localized the rearrangement breakpoints of 3D26A-3, 8B-8, and 7B5-3 by DNA sequencing of 614-4 and 709T-1 and therefore identified the DNA sequences flanking the breakpoint (position II, Fig. 5A) deleted by DNA rearrangement. These DNA sequences bore no resemblance to each other, nor to the heptamer–nonamer recombination sequences occurring at the 3' side of wild-type *D* elements (position II, Fig. 5B). Thus, in nine cases at the 3' side (J_H) and three cases at the 5' side (D_H) where *scid* rearrangement breakpoints were mapped, no consistent DNA sequence features were observed (positions I and II, Fig. 5B). These results indicated that the defect in *scid* gene rearrangement was not due to the selection of altered recombination sequences, which initiated cleavage and rejoining. Instead, we would argue that *scid* breakpoints did not coincide with the initial cleavage point for rearrangement but represented the end point of a random deletion event (see Discussion).

The *D* elements most likely involved in the rearrangement events that generated clones 3D26A-3, 8B-8, and 7B5-3 were identified by DNA sequencing in the genomic regions adjacent to the breakpoint positions. For 3D26A-3 and 8B-8, the *D* element was identified as D_{Q52} , which resides only ~700 bp from the 5' end of the J_H cluster (Fig. 6A). The 614-4 *Eco*RI fragment obtained from germ-line DNA corresponded to the 6.4-kb *Eco*RI fragment that also contained the J_H region. The *D* element within clone 7B5-3 was identified as $D_{FL16.1}$ (Fig. 6B), which is located at the 5'-most end of the D_H region (Kurosawa and Tonegawa 1982; Wood and Tonegawa 1983). In the three cases examined, the closest *D* elements lie between 1153 and 1743 bp removed from the breakpoint position. If normal heptamer–nonamer recognition sequences were utilized for both the *D* and J_H joining step, then in two of these cases, 3D26A-3 and 8B-8, only the D_{Q52} element could possibly have been involved in the gene rearrangement event. It is interesting to note that if the rearrangements initiated concurrently at *J* and *D* heptamer sequences, then the extent of deleted DNA from each heptamer may be asym-

acterized, an ambiguity of 2 and 3 bp in the breakpoints, respectively led to an uncertainty of whether or not any *N* regions of 1–3 bp were added. In the case of clone 8B-8, however, an *N* region of one nucleotide (C) was found that could not be accounted for by the germ-line DNA sequences of the adjoining *D* and *J_H* regions (Fig. 5). The existence of this *N*-region insertion for clone 8B-8 suggested that the *scid* defect did not alter TdT function.

The scid gene encodes a trans-acting factor in DNA rearrangement

The *scid* mutation may directly affect gene rearrangement or it may disrupt lymphoid cells by an unrelated differentiation process such that the Ig and TCR gene rearrangements are altered adversely. Therefore, we ex-

amined the persistence of the *scid* defect in Ab-MLV-transformed pre-B-cell lines to establish the specific role of the mutation in the recombination mechanism. Wild-type Ab-MLV pre-B cell lines generally retain the ability to rearrange exogenous Ig or TCR recombination templates introduced by retroviral infection or transfection (Blackwell and Alt 1984; Lewis et al. 1984, 1985; Akira et al. 1987; Lieber et al. 1987; D. Weaver, unpubl.). *scid* cell lines (8D and 6B) and wild-type cell lines (38B9 and 230-238) were infected with a recombinant retrovirus, DGR (Landau et al. 1987), that contained a rearrangement cassette consisting of a V_{κ} element and two J_{κ} elements (J_a and J_b), the G418 resistance gene *neo*, and a mycophenolic acid (MPA)-resistance gene, *gpt* (Fig. 7). G418 resistance selects for cells that integrate the DGR virus but it is independent of whether or not normal $V-J$ rearrangement occurs. MPA resistance selects for cells that have undergone an inversional $V-J$ rearrangement because the transcriptional orientation of the *gpt* gene in DGR is inverted by rearrangement. The DGR retrovirus integrates via the LTR sequences accurately into chromosomes and has been shown to only rearrange in pre-B cell lines (Landau et al. 1987; Schatz and Baltimore 1988). Therefore, G418-resistant colonies were selected from *scid* and wild-type cell line infections, and ge-

nomeric DNA was isolated, digested with *Bam*HI and *Hind*III, and analyzed by Southern blots using hybridization probe 1 (Fig. 7). The 3.8-kb unrearranged *Bam*HI–*Hind*III DGR fragment is shown in the 230-238 cells (lane 230-238, DGR). $V-J_a$ or $V-J_b$ rearrangement of DGR in this cell line generated primarily two new *Bam*HI–*Hind*III fragments of 1.9 and 1.5 kb (J_a and J_b , respectively, lane 230-238). Additional bands were also detected that presumably arose from secondary rearrangements within the DGR cassette. Secondary rearrangements have been observed previously with DGR and related retroviruses (Lewis et al. 1985; Schatz and Baltimore 1988). An increased frequency of detection of the two primary $V-J$ rearrangements can be selected for in culture with MPA resistance in wild-type cell lines (lane 38B9; see also Landau et al. 1987). In contrast to wild-type rearrangements, all of the G418-resistant *scid* lines showed strikingly different patterns of hybridization (lanes 1–4, 8–11). These G418-resistant *scid* cultures were not clonal, and thus several attempted rearrangement events were represented in each cell culture; yet, significantly, none of the rearrangements were the same as wild-type rearrangements. It is interesting to note that these cell lines are generally not resistant to MPA. This observation is supported by preliminary evidence, indicating that the DGR rearrangements in *scid* cell lines have generated deletions internal to the construct (E.A. Hendrickson and D.T. Weaver, unpubl.). Thus, the *scid* defect associated with DNA rearrangement both persisted in cultured Ab-MLV-transformed cell lines and manifested the mutational effect on substrates introduced at multiple independent chromosomal sites. This experiment strongly suggested that the *scid* gene encodes a *trans*-acting factor that is a primary component of the lymphoid gene rearrangement mechanism.

Figure 7. The *scid* mutation encodes a *trans*-acting factor involved in Ig gene rearrangement. (Top) Established *scid* pre B-cell lines 8D and 6B were infected with DGR, a retrovirus that contains an Ig rearrangement cassette. Genomic DNA from eight independently established cell lines (numbered 1–11) was isolated, digested with *Bam*HI and *Hind*III, and subjected to Southern blot analysis, as described in Experimental procedures. Hybridization probe 1 (solid bar) was used to detect rearrangement events within the DGR rearrangement cassette. The positions of the unrearranged *Bam*HI–*Hind*III fragment of DGR and the V_{κ} – J_a (J_a) or V_{κ} – J_b (J_b) events are shown on the right. (Bottom) The DGR retroviral vector (from the left): the 5'-LTR, the V_{κ} heptamer–nonamer sequences (solid triangle), the *Bam*HI restriction site (B), the V_{κ} (open rectangle), the xanthine–guanine phosphoribosyl transferase gene-coding sequences (*gpt*, hatched rectangle), the J_a and J_b elements complete with their heptamer–nonamer sequences (open rectangle with solid triangle, respectively), the SV40 promoter/enhancer sequences (SV40, stippled rectangle), the *Hind*III restriction site (H), the neomycin gene-coding sequences (*neo*, hatched rectangle), and the 3'-LTR.

Discussion

We have studied Ig heavy-chain DNA rearrangement events that are defective in *scid* mouse pre-B-cell lines. *scid* Ig gene formation was arrested at the *D*–*J_H* rearrangement stage where *D* elements of all three families (*D_{Q52}*, *D_{SP2}*, and *D_{FL16}*) in the entire *D* region were used, suggesting that the chromosomal selection of rearrangement sites was normal. However, extensive deletions usually occurred as a consequence of these rearrangement steps. By obtaining the DNA sequences of the breakpoint junctions of nine *scid* rearrangements, we determined that the joining reactions were defective in every case. No Ig heavy-chain gene products were capable of being synthesized as an outcome of these rearrangements. The structure of the novel breakpoints generated did not correspond to or align with any new or altered heptamer–nonamer recognition sequences in the unrearranged DNA.

The scid gene product is a trans-acting factor of DNA rearrangement

When Ab-MLV *scid* pre-B cell lines were infected with the DGR retrovirus containing an Ig rearrangement cassette, we consistently observed inappropriate deletions of the DGR DNA (Fig. 7). These data argue that the *scid* mutation is not restricted to the endogenous sites of lymphoid gene rearrangement because DGR integration most likely occurs randomly in the genome, and many independent events were observed. Also, we can conclude that the *scid* mutation is cell autonomous because the mutational effect persisted in isolated *scid* pre-B-cell lines. Every rearrangement, either of the endogenous Ig genes or of DGR, was defective, which strongly indicated that the *SCID* gene encodes a *trans*-acting factor that is directly involved in all normal DNA rearrangements, rather than any other lymphoid differentiation function. Therefore, we would predict that the appropriate differentiation of *scid* pre-B cells to surface-Ig⁺ B cells could occur solely by the introduction of normally rearranged Ig μ and κ genes, circumventing the need for the endogenous gene rearrangement steps.

scid rearrangements are stopped at the D–J_H stage

Our data demonstrate that the defective *scid* rearrangements are stopped at the *D*–*J_H* stage; the first stage of Ig heavy-chain gene rearrangement. No light-chain rearrangement or evidence for rearrangement of Ig *V_H* regions into the breakpoint junction fragments was observed. A *V_H81X*-specific hybridization probe (the 3'-most member of the *V_H* cluster) displayed the same intensity of hybridizing *Eco*RI fragments in all the *scid* cell lines (data not shown). Southern blot mapping of the junction fragments (Fig. 4) demonstrated that the 5' region of the clones originated from the *D* region. In two cases, the analysis could not distinguish between *D* or *V* incoming sequences, because the region of hybridization to the *scid* probe was deleted in all of the wild-type Ab-

MLV cell lines used in the assay. In both of these cases (3D26A-3 and 8B-8), the defective rearrangement occurred in the *D_{Q52}–J_H* *Eco*RI fragment (Fig. 6A); they were therefore aberrant *D*–*J_H* joining events. By comparison, in wild-type Ab-MLV pre-B cell lines derived from the bone marrow compartment, ~80% of the Ig heavy-chain alleles have undergone *VDJ_H* rearrangements (Alt et al. 1984). Wild-type Ab-MLV pre-B cells frequently contain *VDJ_H* rearrangements on both alleles, or consist of one *VDJ_H* functionally rearranged allele that produces μ protein and an allelically excluded second allele at the *DJ_H* state. We and others have observed high frequencies of Ig heavy-chain alleles unrearranged or at the *DJ_H* stage in Ab-MLV pre-B-cell lines from μ transgenic mice that are presumably due to allelic exclusion by the μ transgene (Rosconi and Kohler 1985; Weaver et al. 1985; Storb et al. 1986). Therefore, the *scid* data suggested the interesting possibility that accurate *D*–*J_H* rearrangement may be a necessary event for the continuation of the rearrangement and/or differentiation process. *DJ_H* rearrangements have been shown to produce *DJ_H* μ mRNA and D μ proteins in several normal Ab-MLV cell lines, and it has been postulated that production of this molecule may stimulate further gene rearrangement events (Reth and Alt 1984).

One property of the *scid* mutation is that it is somewhat leaky in that a small percentage of the animals can develop an oligoclonal B-cell population with age (Bosma et al. 1983). It is interesting that no joining event we characterized in the *scid* cell lines encoded production of a viable *DJ* μ protein or a full-length μ (*VDJ*) protein on the DNA sequence level. In addition, none of the 12 *scid* Ab-MLV cell lines isolated in this study produced either D μ or μ protein, as determined by metabolic labeling with [³⁵S]methionine, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis (D. Weaver, unpubl.). In 8B-8, the breakpoint occurred within 3 nucleotides of the 3' end of the *J_H* heptamer, which is similar to a normal rearrangement, but the other side of the breakpoint contained no Ig coding sequence. The nearest *D* element still on the chromosome in the 8B-8 rearrangement is ~19 kb, 5' distant (Wood and Tonegawa 1983), and therefore no D μ or μ protein could be synthesized from it. Yet many of the *scid* rearrangements we have examined do not destroy all of the *J_H* and *D* regions, making secondary rearrangement events possible that may accidentally append the appropriate sequences together. Presumably, there is a good selection *in vivo* for this type of low-frequency event, which may explain why the *scid* mutation has a leaky phenotype even though it has a severe recombination defect.

Initiation of recombination by recognition of heptamer–nonamer sequences

Wild-type lymphoid gene rearrangement events are directed by conserved heptamer–nonamer DNA sequences such that both the site of cleavage and rejoining of DNA strands occur flanking these diagnostic ele-

ments. These heptamer–nonamer DNA sequences alone are sufficient to signal DNA rearrangement in exogenously added DNA (Akira et al. 1987). We investigated here whether this recognition phase of the rearrangement process was defective in *scid* pre-B cells. Our data support the notion that the normal heptamer–nonamer sequences are recognized in *scid* pre-B cells and that this recognition process in and of itself is not responsible for the *scid* deletions. An altered heptamer–nonamer motif was not found in the DNA regions flanking the breakpoints of the nine *scid* rearrangements, nor was there DNA homology between the flanking sequences themselves (Fig. 5). In addition, in the three cases where both the *D*- and *J*-region DNA sequences flanking the breakpoint were determined, there was no homology observed between *D*- and *J*-region DNA. If random recognition and cleavage steps occurred in the genomic *EcoRI* fragment to initiate recombination, then it would have been expected that some of the breakpoints would have been positioned in the region 5' to the J_H cluster of the *EcoRI* fragment. However, the positions of nine *scid* breakpoints in the genomic 6.4-kb *EcoRI* fragment all resided on the 3' side of the J_{H1} heptamer element (Fig. 2). Thus, the placement of the *scid* breakpoints is best explained not as representing the initial recognition or cleavage points in the rearrangement event, but as the consequence of variable deletions once the cleavage reaction has taken place. We currently are testing directly whether heptamer–nonamer recombination signals are required to initiate the aberrant rearrangements by introducing into *scid* pre-B cells recombinant retroviruses consisting of Ig rearrangement cassettes containing either wild-type heptamer sequences or heptamer mutations that destroy normal rearrangements (E. Hendrickson and D. Weaver, in prep.).

Normal rearrangement is disrupted at the joining of strands in the *scid* mutation

In one model for Ig recombination, the two gene elements (*D* and J_H) are brought in close proximity, the heptamer–nonamer sequences of the *D* and *J* elements are recognized and cleaved, and a double-strand exonuclease digests either end of the DNA for a few nucleotides (Alt and Baltimore 1982). TdT may then add a few nucleotides to the 3' strand of either end (generating *N* regions). These 3' overhanging ends may then be filled in by a DNA polymerase with a ligase activity finally covalently joining the strands. Of all of these enzymatic functions thought to be needed to generate a functional *D*–*J* joint, we have evidence only for TdT activity. The identification of an *N* region in clone 8B-8 suggested that this activity was not affected in *scid* mice. Our data are most consistent with the *scid* mutation either causing an increase in the activity of the exonuclease or a decrease in activity of the ligase function. If the rejoining of DNA is defective in *scid* mice by an impaired ligase function, the mutation might allow otherwise unrelated cellular nucleases the opportunity to attack the cleaved strands before they could be resealed.

It is also possible that the *scid* mutation encodes a general recombination or repair defect and may not be a lymphoid-specific function. For example, the *SCID* gene may have a parallel phenotype for Ig rearrangement as the *RAD52* gene has for mating-type switching in *Saccharomyces cerevisiae*. Mating-type switching is initiated at a defined DNA position in the MAT locus by the HO endonuclease. In *rad52* yeast strains during mating-type switching, the double strand breaks generated by the activity of HO protein at the normal site are lethal events and frequently give rise to variable deletions (Weiffenbach and Haber 1981; Weiffenbach et al. 1983). Although *rad52* was characterized with a site-specific mating-type switching defect, it is believed to be a protein used in general recombination as well. By analogy, we are investigating this possibility for *scid* by inspecting the properties of *scid* pre-B cells after X-ray or methylmethane sulfonate treatments, both of which generate double-stranded breaks randomly in DNA.

Experimental procedures

Cells and virus

Bone marrow from a 10-week old homozygous *scid* mouse was removed and transformed with Ab-MLV (P160) by standard procedures (Rosenberg and Baltimore 1976; Weaver et al. 1985). Transformed colonies of pre-B cells were removed from soft agar plating and expanded in liquid media. Equivalent numbers of *scid* Ab-MLV cell lines were isolated, as compared to the standard yield from wild-type mouse bone marrow.

DGR virus was maintained in a ψ 2-producer cell line (ψ -DGR; Landau et al. 1987). *scid* pre-B cell lines 8D and 6B (1×10^6 cells) were infected by cocultivation with ψ -DGR cells (5×10^6 cells) for 48 hr prior to selection in media containing 2 mg/ml G418 for 3 weeks.

Southern blots

Genomic DNA was isolated for *scid* and wild-type Ab-MLV transformed pre-B cell lines (1×10^8 to 2×10^8 cells) by treatment in 400 μ g/ml of proteinase K, 1% SDS, 10 mM Tris-HCl (pH 8.0), 50 mM EDTA, and 100 mM NaCl for >6 hr at 37°C. The samples were then extracted once with an equal volume of phenol, followed by one extraction with an equal volume of CHCl_3 . The DNA was then precipitated with an equal volume of isopropanol, spooled from solution, and resuspended at a concentration of 1 mg/ml in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. Ten micrograms of genomic DNA was digested with restriction enzymes, fractionated on 0.8% agarose gels, prepared and transferred to nitrocellulose or Zetabind paper in $20 \times \text{SSC}$, and baked in a vacuum oven (Maniatis et al. 1982). Prehybridization was conducted for >4 hr at 42°C in 50% formamide, $10 \times$ Denhardt's solution, $5 \times \text{SSPE}$, 1% SDS, and 500 μ g/ml denatured herring sperm DNA. Hybridization probes were prepared from isolated restriction fragments, as specified in the figure legends, using mixed oligonucleotide primers (Boehringer Mannheim) and Klenow fragment DNA polymerase (Feinberg and Vogelstein 1984). Hybridizations were conducted for 36 hr at 42°C in 50% formamide, $1 \times$ Denhardt's solution, $5 \times \text{SSPE}$, 1% SDS, and 100 μ g/ml denatured herring sperm DNA with the hybridization probes (sp. act. 10^9 cpm/ μ g). Blots were washed for 15 min at room temperature in $2 \times \text{SSC}$, 0.1% SDS, followed by three consecutive washes for 30 min each at 68°C in $2 \times \text{SSC}$, 0.1% SDS.

Cloning of breakpoint fragments

Forty micrograms of genomic DNA from nine Ab-MLV *scid* pre-B cell lines was digested with *EcoRI* and fractionated in 0.8% agarose gels. *EcoRI*-cleaved DNA size fractions containing the *scid* rearrangements were determined by a previous Southern blot (i.e., Fig. 1). One relevant size fraction per cell line was isolated from the preparative gel by silica glass powder binding in NaI (Vogelstein and Gillespie 1979). A phage λ gt10 library was then prepared from the size-selected DNA for each cell line (Maniatis et al. 1982). Phage libraries ($\sim 2 \times 10^4$ to 10×10^4 recombinants per size selection library) were screened using a 677 nucleotide-*XbaI*-*EcoRI* fragment (3' probe, Fig. 1) hybridization probe (Maniatis et al. 1982). Phage DNA was isolated by standard miniprep procedures, and the *EcoRI* inserts were subcloned into pUC19 to generate the *scid* clones depicted in Figure 2.

DNA sequencing of the J_H region breakpoint junctions

The breakpoint junction of *scid* rearrangements in the J_H region were characterized by extensive restriction enzyme mapping and comparison to the known pattern for the germ-line 6.4-kb *EcoRI* fragment containing the J_H region. A small DNA restriction fragment spanning the breakpoint junction for each clone was radiolabeled at its 5' termini by the sequential treatment with calf intestinal phosphatase and then polynucleotide kinase (Boehringer Mannheim) in the presence of [γ - 32 P]ATP. Radiolabeled DNA was then digested with a second restriction enzyme to yield two uniquely radiolabeled DNA fragments that were separated on a 1.0% agarose gel. Radiolabeled DNA was recovered from the gel by elution onto NA45 paper (Schleicher and Schuell). The radiolabeled fragments were subjected to DNA sequencing (Maxam and Gilbert 1980) and fractionated in 6% polyacrylamide-8 M urea sequencing gels using 0.2 to 1.0-mm wedge-shaped spacers. Gels were fixed for 1 hr in 10% glacial acetic acid, 12% methanol, vacuum-dried, and exposed for autoradiography.

Isolation and DNA sequencing of D region breakpoint junctions and flanking D elements

Hybridization probes were prepared from 0.15-kb *PvuII*-*OxaNI* fragment of 3D26A-3 (see Fig. 6A) and from a 1.0-kb *HindIII* fragment of 7B5-3 (Fig. 6B). We screened a phage λ EMBL3 genomic library containing partial *MboI* fragments of germ-line DNA from a BALB/c mouse (Winoto et al. 1985) independently with each hybridization probe (Maniatis et al. 1982). DNA from individual positive phage was isolated after a tertiary screen and then digested with the restriction enzyme *EcoRI*. The resulting DNA fragments were analyzed by Southern blotting as detailed above, and the positively hybridizing *EcoRI* fragments were subcloned into the *EcoRI* site of pUC19 to generate clones 614-4 (homologous to the 3D26A-3 probe) and 709T-1 (homologous to the 7B5-3 probe).

A 450-nucleotide *PstI* fragment spanning the breakpoint junction of 709T-1 DNA (Fig. 6B) was subcloned into the *PstI* site of M13mp19. Subclones of both orientations were sequenced according to Sanger et al. (1977) using the Sequenase sequencing kit (US Biochemicals). The 0.9-kb *BamHI* fragment of 709T-1, which contains the *D* element, was subcloned into the *BamHI* site of pUC19. A series of plasmids containing overlapping, nested deletions into the insert region were generated using the Erase-a-Base system (Stratagene). Plasmid DNAs were purified from minipreps by an alkaline lysis procedure (Maniatis et al. 1982), and 3 μ g plasmid DNA was further purified

with glass beads and NaI. Plasmid DNA (1.5 μ g) was denatured in 0.2 N NaOH for 5 min, EtOH-precipitated in 0.33 M NH_4OAc , resuspended in H_2O , and hybridized to either the Universal or Reverse DNA sequencing primers (US Biochemicals), and sequenced with the Sequenase kit. The breakpoint DNA sequence and the *D* element for 614-4 were determined by subcloning the 2.4-kb *BamHI* fragment encompassing both of these elements (Fig. 6A) into the *BamHI* site of pUC19. As described above, a series of plasmids containing overlapping, nested deletions were generated and sequenced using the Erase-a-Base and Sequenase protocols.

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E A Hendrickson, D G Schatz and D T Weaver

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