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Abnormal Deletions in the T-Cell Receptor δ Locus of Mouse Thymocytes

SUZANNE M. FISH AND MELVIN J. BOSMA*

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

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Separate genetic elements (V, D, and J) encode the variable regions of lymphocyte antigen receptors. During early lymphocyte differentiation, these elements rearrange to form contiguous coding segments (VJ and VDJ) for a diverse array of variable regions. Rearrangement is mediated by a recombinase that recognizes short DNA sequences (signals) flanking V, D, and J elements. Signals flank both the 5′ and 3′ sides of each D element, thereby allowing assembly of a functional VDJ gene. However, in rearrangements involving the Dδ2 and Jδ1 elements of the mouse T-cell receptor δ (TCR δ) locus, we unexpectedly found that the Dδ2 element and a portion of its 5′ signal are often deleted. Approximately 50% of recovered Dδ2 to Jδ1 rearrangements from thymocytes of adult wild-type mice showed such deletions. An additional 20% of the rearrangements contained standard Dδ2-Jδ1 coding junctions but showed some loss of nucleotides from the 5′ Dδ2 signal. This loss was clearly associated with another event involving a site-specific cleavage at the 5′ signal/coding border of Dδ2 and rejoining of the modified signal and coding ends. The abnormal loss of Dδ2 and a portion of the 5′ Dδ2 signal was infrequently observed in Dδ2-to-Jδ1 rearrangements recovered from neonatal mice. The possible basis and significance of this age-dependent phenomenon are discussed.

During early lymphocyte differentiation, separate genetic elements (V, D, and J) recombine in combinatorial fashion to form contiguous coding segments (VJ and VDJ) for the variable regions of antigen receptors (reviewed in references 32, 54, and 64). The activity responsible for this recombination recognizes short DNA sequences (signals) flanking V, D, and J coding elements. The signals consist of a conserved heptamer and nonamer separated by a 12- or 23-bp spacer and are henceforth referred to as 12 or 23 signals, respectively. Recombination of any two coding elements requires that they be flanked by signals with different-length spacers (12/23 rule). The standard reaction is believed to involve double-strand cleavages at the heptamer/coding borders followed by rejoining of the DNA ends in a new configuration, i.e., coding to coding and signal to signal to form coding and signal junctions, respectively. Coding (but not signal) ends are usually modified prior to joining and show base loss and/or addition. The base additions include templated, palindromic (P) additions to unmodified coding ends (29, 39) and nontemplated (N) additions mediated by the enzyme terminal deoxynucleotidyltransferase (1, 14, 27, 31).

Not all V(D)J joining reactions yield the standard coding and signal junctions (32). Coding ends may sometimes be rejoined to the signal of another coding element to form a hybrid junction (35, 42). In recombinase-active lymphoid cell lines transfected with plasmid V(D)J recombination substrates, the frequency of plasmid recombinants with hybrid junctions can be as high as 25% (35). Another nonstandard V(D)J junction seen with plasmid and retroviral recombination substrates is an open-and-shut junction. After cleavage at a given signal/coding border and loss and/or addition of nucleotides (usually only at the coding end), the signal and coding sequences are rejoined (18, 34, 35). The incidence of open-and-shut junctions with plasmid substrates is ~1% (34). Apparent examples of hybrid junctions and open-and-shut junctions have been observed occasionally in endogenously rearranged antigen receptor genes, but the incidence of these junctions is unknown (8, 11, 50, 51).

In this study, we report a high frequency of unusual recombination junctions in rearranged T-cell receptor δ (TCR δ) genes of thymocytes from adult mice. The δ locus is one of four TCR loci (α, β, γ, and δ) and appears to be the first to undergo rearrangement in developing mouse embryos (7, 9, 10). It is located within the TCR α locus, such that the reciprocal product of a Vα-to-Jα rearrangement is an excised circle containing TCR δ elements (9, 10, 45). The TCR δ locus contains two D (Dδ1 and Dδ2), two J (Jδ1 and Jδ2), and at least six V elements (11). The 5′ and 3′ sides of the D elements are flanked by 12 and 23 signals (12/Dδ/23); V and J elements are flanked on their 3′ and 5′ sides, respectively, with 23 and 12 signals (Vδ/23 and 12/Jδ). There is no strict order of rearrangement of these elements; D-J, V-D, and D-D coding junctions have been recovered from the TCR δ locus of thymocytes and thymocyte hybridomas (10).

We recovered Dδ2-to-Jδ1 rearrangements from thymocytes of neonatal and adult mice by using PCR. Most rearrangements (>80%) from neonatal mice were normal and contained standard Dδ2-Jδ1 recombination junctions, whereas approximately half of the rearrangements from adult mice were missing Dδ2 and nucleotides from the 12/Dδ2 signal. Deletions into the 12/Dδ2 signal would prevent usage of this signal for recombination of the upstream Dδ1 or Vδ coding elements. This would preclude the assembly of a functional Vδ-Dδ2-Jδ1 (or Vδ-Dδ1-Dδ2-Jδ1) gene. We refer to these deletions as abnormal Dδ2-associated deletions and suggest that some may involve an open-and-shut event at the 12/Dδ2 border. Clear evidence of open-and-shut 12/Dδ2 junctions with loss of 12 signal nucleotides was seen in about 20% of the recovered Dδ2-to-Jδ1 rearrangements from adult mice. Two such junctions were also recovered from δ loci with an unrearranged Dδ2 element, indicating that open-and-shut events may occur before the joining of Dδ2 to Jδ1. Possible mechanisms for the observed Dδ2-associated deletions are discussed.

* Corresponding author. Mailing address: Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111. Phone: (215) 728-3630. Fax: (215) 728-2412.

MATERIALS AND METHODS

Mice. C.B-17 (wild-type) and C.B-17*scid/scid* (scid) mice were obtained from the specific-pathogen-free animal facility of Fox Chase Cancer Center. Thymuses were obtained from neonatal mice (1 to 2 days old) and adult mice (2 to 7 months old).

DNA. DNA was isolated from pooled thymus tissue in the manner described by Blin and Stafford (4) by using modifications of Sambrook et al. (53). DNA was also obtained from thymocytes of a single mouse or thymocytes pooled from several mice by using GeneReleaser (Bioventures, Murfreesboro, Tenn.).

Pulsed-field gel electrophoresis. DNA was prepared in agarose plugs as described previously (53) with minor modifications. Equal volumes of thymocytes in phosphate-buffered saline and 1.2% Incert agarose (FMC, Rockland, Maine) were mixed. The DNA was then purified by addition of 0.4 M EDTA (pH 8.0), 1% Sarkosyl, and 2 mg of proteinase K per ml and incubation at 55°C for 48 h. Plugs containing DNA were then equilibrated with 40 μg of phenylmethylsulfonyl fluoride per ml–10 mM Tris (pH 8.0)–10 mM EDTA and stored in 0.5 M EDTA (pH 8.0). Plugs containing DNA from 3×10^5 thymocytes were equilibrated with 0.5× TAE (53)–10 mM EDTA and integrated into a 1% 1:1 Fast Lane:GTG agarose gel (FMC). Electrophoresis was performed with a model 2604 Waltzer II rotating stage apparatus (Tribotics, Oxford, United Kingdom) at 16°C, 160 V, and a 49-s switch time for 18 h in 0.5× TAE. After equilibration with 1× *Taq* buffer, DNA remaining in the Incert plug (high-molecular-weight DNA) was used as template in PCR amplification as described below.

PCR. Nested sets of primers located ~100 bp from each coding element (5′ Dδ2^{ext} [5′ CTTAAGTACCCAGGTCA AGTCT 3′], 5′ Dδ2^{int} [5′ AAAAGATCTGGCCTGAAC TAACTGCCA 3′], 3′ Jδ1^{ext} [5′ AAAAAGCTTACTCAA CACGACTGGA 3′], and 3′ Jδ1^{int} [5′ GGAAGCTTACTTC CAACCTCTTTAGGT 3′]) were used to amplify Dδ2-Jδ1 recombination products (7). Approximately 3 μg of DNA (or DNA from 5×10^5 cells if GeneReleaser is used) and external primers (each at 0.5 μM) were incubated at 95°C for 5 min in a solution containing 10 mM Tris (pH 8.3 at room temperature), 50 mM KCl, 3.0 mM $MgCl_2$, 0.01% gelatin, and 1 U of AmpliTaq (Perkin Elmer-Cetus, Norwalk, Conn.). DNA amplification was carried out for 20 cycles (30 s at 94°C, 30 s at 55°C, and 2.5 min at 72°C), followed by a final 5-min incubation at 72°C. A second amplification was then carried out for 20 to 40 cycles with a 1:50 dilution of the above reaction mixture, internal primers, and an annealing temperature of 55°C. The above 5′ Dδ2^{ext} and 5′Dδ2^{int} primers, together with two primers located 100 to 200 bp downstream of Dδ2, 3′ Dδ2^{ext} (5′ CCGAGATCTGTAACATGTTTTACCCAG 3′) and 3′ Dδ2^{int} (5′ GCAAGTGGAGGTCAAAGCTTGTCC 3′), were used to recover 12/Dδ2/23 sequences with open-and-shut junctions at the 12/Dδ2 border and sequences with a missing Dδ2 and joined 12/Dδ2 and Dδ2/23 signals. PCR conditions were as above except the annealing temperature was 57°C in the first PCR amplification and the $MgCl_2$ concentration was 2.0 mM in the second PCR amplification. Primers 140 and 40 bp from Dβ1.1 and Jβ2.6 elements, respectively, were used to amplify Dβ1.1-Jβ2 recombination products. These primers (Dβ1.1 [5′ GGGGAGCTCGCATCT TACCACCAC 3′], Jβ2^{ext} [5′ GGGGAATTCGATTTCCCTC CCGGAGA 3′], and Jβ2^{int} [5′ CGCGGATCCAAACTACTC CAGGGA 3′]) are described by Petrini et al. (47). PCR conditions were the same as those for amplifying Dδ2-Jδ1 recombination products except that the concentration of $MgCl_2$ was reduced to 2.0 μM. Also, the first PCR amplification consisted of 5 cycles with an annealing temperature of 55°C followed by 15 cycles with an annealing temperature of 65°C. When the Jβ2^{ext} primer was replaced with the Jβ2^{int} primer, another amplification was performed with a 1:50 dilution of the first reaction mixture, using annealing temperatures of 50°C for 5 cycles and 65°C for 25 cycles.

Cloning and screening PCR products. PCR products were ethanol precipitated, digested with restriction enzymes specific for recognition sites present in the primers, and ligated into pGem-3Z (Promega, Madison, Wis.). The ligation reaction mixture was electroporated into DH5α cells. Bacterial colonies were lysed (16) and screened for the presence of plasmid containing TCR sequences by using PCR and the above primers (3′ Dδ2^{int} was substituted with another 3′ Dδ2^{int} primer, 5′ AGGGCAGGCTGCGGGCTGTGTTTAC 3′). Rapid screening of products for alterations at the 12/Dδ2 border was performed by digestion with *Sau*3AI. The sequence recognized by *Sau*3AI, GATC, spans the 12/Dδ2 border. Insertion or deletion of nucleotides at this sequence will abolish the *Sau*3AI site.

Sequencing. Plasmids purified with Magic Miniprep (Promega) were sequenced with a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) by using M13 universal and/or M13 reverse primer.

RESULTS

Abnormal Dδ2-associated deletions in wild-type mice: apparent increased frequency in adult mice. Dδ2-to-Jδ1 rearrangements were PCR amplified by using primers located approximately 100 bp upstream and downstream of Dδ2 and Jδ1, respectively. The PCR products were then cloned and sequenced. As indicated in Fig. 1, most of the Dδ2-to-Jδ1 rearrangements recovered from neonatal mice contained standard coding junctions showing moderate base loss at Dδ2 and/or Jδ1 but no N additions (group I). The relative absence of N addition in the recombination junctions is expected (2, 5, 12, 13, 15, 29) and reflects a low synthesis of terminal deoxynucleotidyltransferase in recombinase-active cells of neonatal mice (52). Two rearrangements appear abnormal (group II) and show a deletion of Dδ2 and a portion of the 12/Dδ2 signal.

Figure 2 shows that, in contrast to neonatal mice, about half the Dδ2-to-Jδ1 rearrangements recovered from adult mice contained a deletion of Dδ2 and some 12/Dδ2 signal nucleotides (group II). Most of the rearrangements in this group show some base loss at Jδ1 and contain N additions; two contain short P additions. Rearrangements with standard Dδ2-Jδ1 coding junctions are listed in group I. Most of the junctions in this group contain N and/or P additions. Five of the rearrangements show an open-and-shut junction at their 12/Dδ2 border. All of these open-and-shut 12/Dδ2 junctions show loss of nucleotides at the 12 signal and unmodified Dδ2 coding ends with dinucleotide P additions (AT).

Open-and-shut 12/Dδ2 junctions with loss of nucleotides at the 12 signal were also recovered from TCR δ loci containing an unrearranged Dδ2 element. PCR-amplified 12/Dδ2/23 sequences with open-and-shut junctions at the 12/Dδ2 border were enriched prior to cloning by using *Sau*3AI to restrict products with germ line sequence at the 12/Dδ2 border; the resistant products were gel purified and cloned. The cloned sequences obtained are shown in Fig. 3. Two clones (Fig. 3A) show nucleotide loss and N additions at the 12/Dδ2 border with retention of the Dδ2 element. The remaining clones (Fig. 3B) show loss of Dδ2 and fusion of the two Dδ2 signals; two

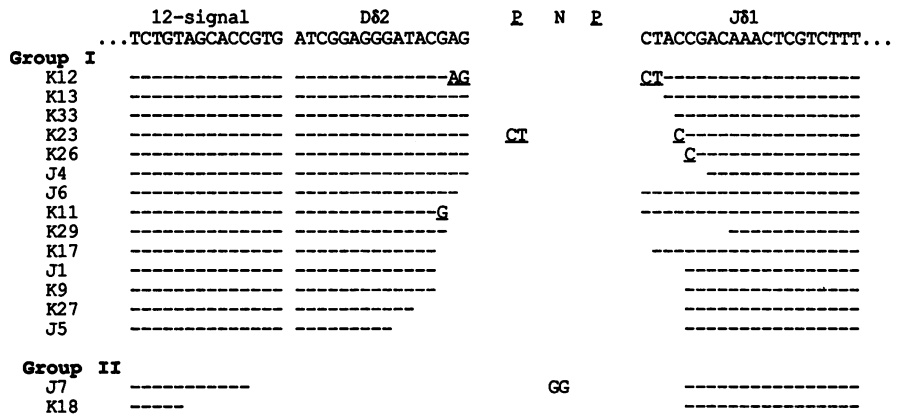

FIG. 1. Dδ2-to-Jδ1 rearrangements recovered from thymocytes of neonatal wild-type mice. The germ line sequence of the 12/Dδ2 signal, Dδ2, and Jδ1 is shown at the top; below are sequences of cloned rearrangements. The dashes correspond to germ line nucleotides. P additions are underlined and shown under "P." N nucleotides are shown under "N." Group I is composed of clones with standard Dδ2-Jδ1 coding junctions. Group II is composed of clones in which the entire D coding region and some 12/Dδ2 signal nucleotides have been deleted. Some rearrangements were isolated more than once from a single DNA preparation and were assumed to represent the same recombination event. The J and K series of clones were from independent pools of thymocyte DNA. In a number of clones (K12, K23, K26, and K11), the assignment of the last one or two nucleotides at Dδ2 and/or Jδ1 is arbitrary, since these nucleotides could represent Dδ2- or Jδ1-derived P additions. Such nucleotides are shown explicitly under the germ line sequence and are underlined. Note that dinucleotide P additions at Dδ2 and Jδ1 create a 4-nucleotide homology (AGCT); use of this homology could account for the Dδ2-Jδ1 junctions in clones K12 (this figure); M10 (see Fig. 2); and C27, A4, and B13 (see Fig. 5). Terminal nucleotides that could have derived from Dδ2 or Jδ1 have been arbitrarily assigned to Dδ2, as, for example, in clones J1 and K9. Arbitrary assignments of terminal nucleotides to Dδ2 (or the 12/Dδ2 signal) have been made for clones C28, B12, and B7 (see Fig. 5) and G12, E2, G15, and F18 (see Fig. 6).

(ND8 and QD2) contain perfect signal junctions, and two (QD1 and QD7) contain imperfect signal junctions with nucleotide loss and addition at the signal ends.

Detection of abnormal Dδ2-associated deletions in high-molecular-weight DNA from adult mouse thymocytes. The abnormal Dδ2-associated deletions could derive from δ loci that have been excised from the chromosome following Vα-to-Jα rearrangement (45) and/or from nonexcised chromosomal δ loci. Most excised circular DNA containing the δ locus could be expected to range between 50 and 1,000 kb on the basis of a comparison of the murine and human α and δ loci (see reference 30 and references therein). Therefore, in an attempt to exclude or minimize the presence of circular DNA in the PCR amplification, we isolated very-large-molecular-size DNA (>2,000 kb) from adult thymocytes by means of pulsed-field gel electrophoresis. Using this DNA, we again found that about half of the recovered Dδ2-to-Jδ1 rearrangements showed a deletion of Dδ2 and some 12/Dδ2 signal nucleotides (Fig. 4, group II); the remaining rearrangements contained standard Dδ2-Jδ1 coding joints (Fig. 4, group I). Note that two of the eight rearrangements in group I have an open-and-shut junction at their 12/Dδ2 borders (P16 and P20) and that there is an unusual loss of nucleotides from the signal ends. The results in Fig. 4 and those obtained with scid mice

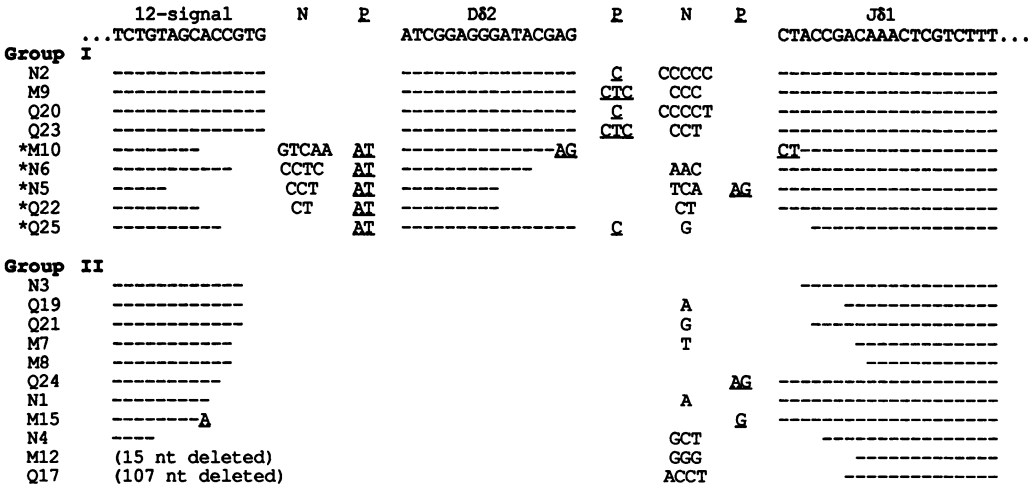

FIG. 2. Dδ2-to-Jδ1 rearrangements recovered from thymocytes of adult wild-type mice. See Fig. 1 for a general description. Deletions of Dδ2 that extend more than 14 nucleotides (nt) into the 12 signal are indicated in parentheses. Clones prefixed with an asterisk (M10, N6, N5, Q22, and Q25) contain open-and-shut junctions at the 12/Dδ2 border. The M series of clones was from thymocyte DNA of a 6-month-old mouse; the N and Q series were from different thymocyte DNA preparations of 4-month-old mice.

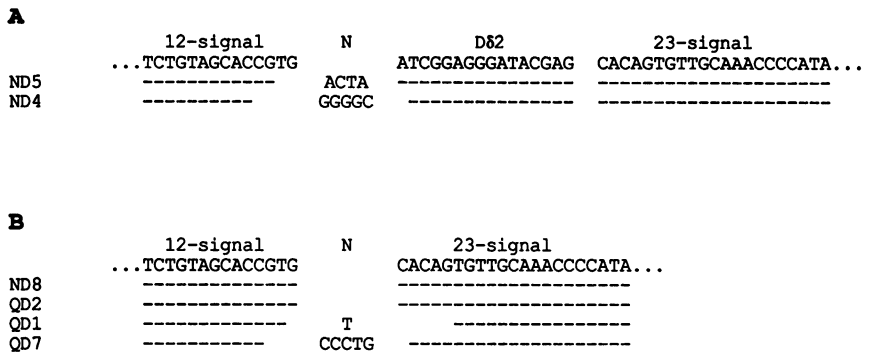

FIG. 3. Recovery of Dδ2-associated deletions with retention of the Dδ2/23 signal in thymocytes of adult wild-type mice. The germ line sequence of the 12/Dδ2 signal, Dδ2, and the Dδ2/23 signal are shown. See Fig. 1 for general description. Clones were derived from DNA preparations N and Q, described in the legend to Fig. 2. (A) Clones with open-and-shut 12/Dδ2 junctions. (B) Clones showing loss of Dδ2 and joined 12/Dδ2 and Dδ2/23 signals.

(see Fig. 6) suggest that chromosomal (nonexcised) DNA may be the major source of abnormal Dδ2-associated deletions. Such deletions could therefore potentially affect the generation of γδ cells.

Abnormal Dδ2-associated deletions in scid mice: apparent increased frequency in adult mice. The TCR α locus, whose rearrangement follows that of δ, γ, and β in normal developing T cells (9, 17, 46), does not appear to be transcribed or rearranged in scid thymocytes (57). Accordingly, excised DNA molecules bearing the δ locus as a result of rearrangement at the α locus would be rare in scid thymocyte DNA, and most Dδ2-to-Jδ1 rearrangements recovered from scid thymocytes should be representative of chromosomal events. As previously shown, normal Dδ2-to-Jδ1 rearrangements can be readily recovered from scid thymocytes by PCR amplification (7). Thus, it was of interest to test whether scid mice show an age-dependent increase in the frequency of abnormal Dδ2-associated deletions similar to that for wild-type mice.

As shown in Fig. 5, the majority of Dδ2-to-Jδ1 rearrangements from thymocytes of neonatal scid mice contained standard Dδ2-Jδ1 coding junctions (group I). Surprisingly, almost half of the rearrangements in this group (5 of 12) show N additions. This is in contrast to 0 of 14 rearrangements from neonatal wild-type thymocytes (Fig. 1, Group I). Note the unusually long P additions at both coding ends in clone D21. There is also a long P addition in clone B11. Long P additions have been previously noted in scid coding joints (24, 28, 55). Five rearrangements show abnormal deletions of Dδ2 and a portion (or all) of its 12 signal (group II). In two members of this group, the deletions extend more than 60 nucleotides; one clone also contains a large (36-nucleotide) deletion into Jδ1. Deletions of this size and larger are commonly seen in rearranged T-cell receptor and immunoglobulin loci of transformed scid lymphocytes (19, 25, 38, 44, 55, 56, 58).

Most of the Dδ2-to-Jδ1 rearrangements recovered from thymocytes of adult scid mice showed abnormal deletions of Dδ2 along with some 12/Dδ2 signal nucleotides (Fig. 6, group II). In three such clones (G15, F18, and F19), the deletions extend >45 nucleotides into the region upstream of Dδ2; in clone F18, the entire Jδ1 region is also deleted. Retention of Dδ2 coding sequence is seen in five rearrangements (group I), although two of these rearrangements (clones E1 and E7) show a loss of 36 and 52 nucleotides from the end of Jδ1. Note the unusually long P addition in clone E3.

A high frequency of abnormal Dδ2-associated deletions was not observed in a previously reported analysis of Dδ2-to-Jδ1 rearrangements from thymocyte DNA of adult scid mice (7). In the earlier report, only 2 of 12 rearrangements involving Dδ2 and Jδ1 showed a deletion of Dδ2; the other 10 rearrangements had standard Dδ2-Jδ1 coding junctions. The basis for the apparent discrepancy between the previous and present results is not clear. Nevertheless, we have now recovered many

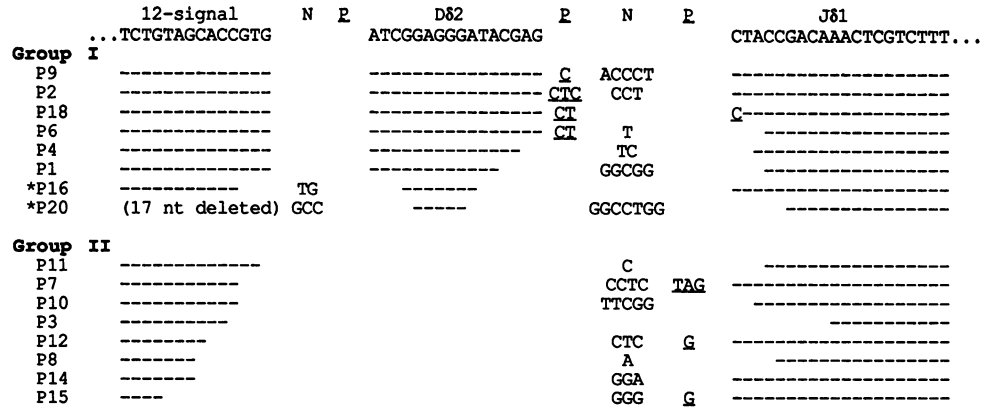

FIG. 4. Dδ2-to-Jδ1 rearrangements recovered from high-molecular-weight DNA in thymocytes of adult wild-type mice. See the legends to Fig. 1 and 2 for a general description. The P series of clones was derived from large-molecular-size DNA (>2,000 kb) that was fractionated by pulsed-field gel electrophoresis. The DNA came from two 7-month-old mice. nt, nucleotides.

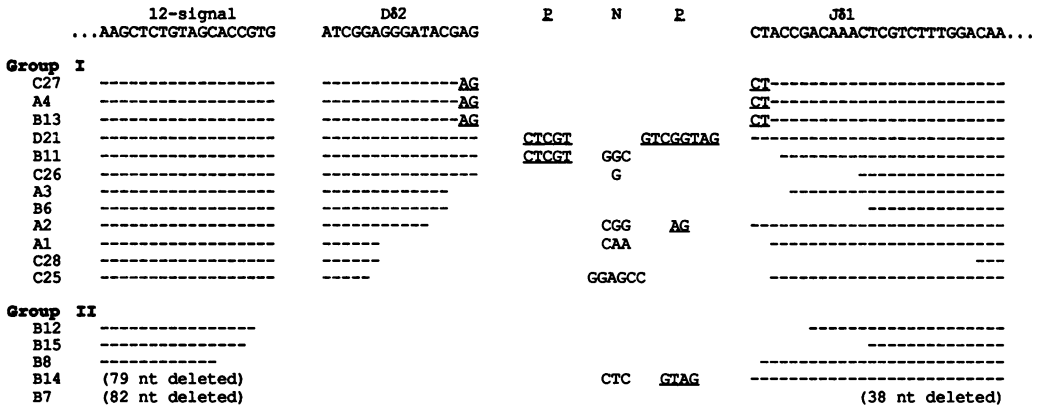

FIG. 5. Dδ2-to-Jδ1 rearrangements recovered from thymocytes of neonatal scid mice. See the legends to Fig. 1 and 2 for a general description. The A, B, C, and D series of clones were each derived from independent DNA preparations of separately pooled scid thymocytes. In the Dδ2-derived P addition of clone D21, the terminal nucleotide (T) could also represent a Jδ1-derived P addition. nt, nucleotides.

additional Dδ2-to-Jδ1 rearrangements from independent samples of neonatal and adult scid mice and have confirmed the age-dependent increase in frequency of abnormal Dδ2-associated deletions (see Table 1, classes D and E).

Low frequency of abnormal Dβ1.1-associated deletions in adult wild-type and scid mice. Although the organization of the TCR β locus differs from that of the TCR δ locus, both of these loci have the same configuration of 12 and 23 signals at their V, D, and J elements. Therefore, if abnormal Dδ2-associated deletions were a general phenomenon and were dependent only on a particular configuration of signals, we would expect to see a high frequency of abnormal D-associated deletions at the TCR β locus as well. To test for this possibility, we PCR amplified, cloned, and sequenced rearrangements involving Dβ1.1, Jβ2.5, and Jβ2.6. As shown in Fig. 7, all rearrangements recovered from adult wild-type mice were normal and contained standard Dβ1.1-Jβ2 coding junctions. The same was true in all but one of eight rearrangements from newborn wild-type mice; the one exceptional rearrangement showed an abnormal deletion of Dβ1.1 along with some 12/Dβ1.1 signal nucleotides (data not shown). In adult scid mice, 9 of 11 rearrangements (Fig. 8, group I) contained standard Dβ1.1-Jβ2 coding junctions. Note the unusually long P additions in clones EC1, EC4, and EC12. Two clones show an abnormal deletion of Dβ1.1 as well as a portion of the 12/Dβ1.1 signal (FC24 and EC2, group II).

It should be noted here that the recovered scid rearrangements shown in Fig. 5, 6, and 8 are not to be taken as representative of the status of TCR β and δ genes in scid thymocytes. On the basis of previous results, most of the thymocytes present in scid mice retain their TCR β and δ loci in germ line configuration (7, 49, 57). Furthermore, the PCR assay used in the present study would exclude rearranged scid alleles with deletions extending more than 200 bp beyond either coding element. Deletions of several hundred base pairs and greater are characteristically seen in transformed scid lymphocytes, as mentioned above.

Overall number of Dδ2-to-Jδ1 rearrangements with normal and abnormal recombination junctions in fetal, neonatal, and adult mice. A summary of the number of rearrangements with normal and abnormal recombination junctions from Fig. 1, 2, and 4 to 8 and from sequence data not shown (Dβ1.1-to-Jβ2 rearrangements from neonatal wild-type mice) is given in Table 1. Also included is a summary of the number of Dδ2-to-Jδ1 rearrangements with and without nucleotide modifications at the 12/Dδ2 border (classes D and E). These two classes were distinguished by a rapid screening of PCR-amplified Dδ2-to-Jδ1 rearrangements for alteration of a

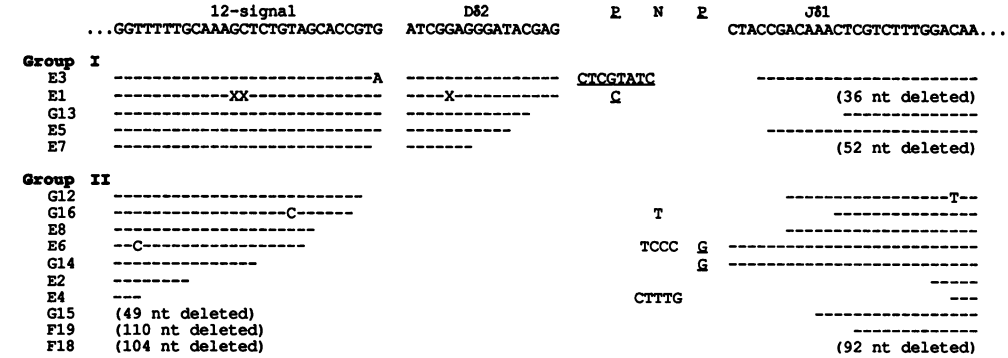

FIG. 6. Dδ2-to-Jδ1 rearrangements recovered from thymocytes of adult scid mice. See the legends to Fig. 1 and 2 for a general description. The E series of clones was from thymocyte DNA of 7-week-old mice; the F and G series were from different thymocyte DNA preparations obtained from individual 10-week-old mice. Nucleotides (nt) different from the germ line sequence are shown explicitly. Ambiguous nucleotides are denoted by an "X." Note that the entire coding region of Jδ1 was deleted in clones E7 and F18.

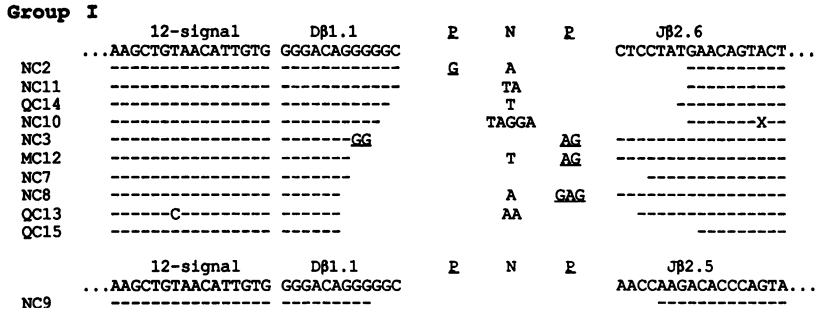

FIG. 7. Dβ1.1-to-Jβ2 rearrangements recovered from thymocytes of adult wild-type mice. The germ line sequence of the 12/Dβ1.1 signal, Dβ1.1, Jβ2.5, and Jβ2.6 are shown at the top; below are sequences of cloned rearrangements. The format is the same as in the earlier figures. Clones were derived from the DNA preparations M, N, and Q as described in the legend to Fig. 2.

*Sau*3AI restriction site spanning the 12/Dδ2 border (see Materials and Methods). For wild-type mice, the frequency of abnormal Dδ2-associated deletions was 2% in 16-day-old embryos, 12% in 1- to 2-day-old neonates, and 65 to 70% in adults. A similar but less striking age-dependent difference was seen between neonatal (27%) and adult (55%) scid mice. In contrast, the frequency of abnormal Dβ1.1-associated deletions appears to be ≤5% on the basis of a much smaller sample of rearrangements from neonatal and adult wild-type mice (classes F and G). In adult scid mice, the frequency of abnormal Dβ1.1-associated deletions (18%) is also much lower than that of Dδ2-associated deletions (55%).

Scid recombination junctions with long P additions (>4 nucleotides) were classified as normal even though P additions this long have not been observed in V(D)J junctions of wild-type mice (41). Junctions with long P additions would still allow for the assembly of a VDJ gene, as opposed to junctions with abnormal D-associated deletions. As noted above, long P additions have been previously reported in scid coding junctions. In these earlier studies (24, 28), 10 to 20% of the scid P additions in VJ and VDJ junctions were >4 nucleotides in length. However, in the present study of DJ junctions, 46% of the scid P additions were longer than 4 nucleotides. Whether this difference is significant is unclear.

DISCUSSION

We have observed an age-dependent increase in abnormal Dδ2-associated rearrangements in thymocytes of wild-type (and scid) mice. Whereas most of the Dδ2-to-Jδ1 rearrangements recovered from neonatal wild-type mice were normal, approximately 65 to 70% of those recovered from thymocytes of adult wild-type mice showed a loss of 12/Dδ2 signal nucleotides. (In many cases there was also a loss of Dδ2.) It was clear that the loss of 12/Dδ2 signal nucleotides in ~20% of the examples resulted from an open-and-shut event at the 12/Dδ2 border. Such a high frequency of open-and-shut junctions has not been reported previously. We discuss open-and-shut events and the possibility that Dδ2-associated deletions involve such events.

Open-and-shut junctions. The only distinguishing feature of open-and-shut junctions is a loss and/or addition of nucleotides at the signal/coding border. Detection of these junctions has therefore depended on special systems and procedures. Open-and-shut junctions were first defined in recombinase-active lymphoid cell lines transfected with extrachromosomal (plasmid) recombination substrates containing two consensus signals (35). Rearrangement of these substrates was assessed by using specially designed screening procedures. A subfraction of the recombinant plasmids recovered from the transfected cell lines were shown to contain open-and-shut junctions at one of the signal/coding borders. Lewis and Hesse (34) extended this work with modified substrates and found that open-and-shut junctions can be recovered at comparable frequency (about 1% of all plasmid recombinants) regardless of whether the substrate contains one or two consensus signals. In substrates with two consensus signals, the frequency of double open-and-shut junctions (at both signal/coding borders) was about four-

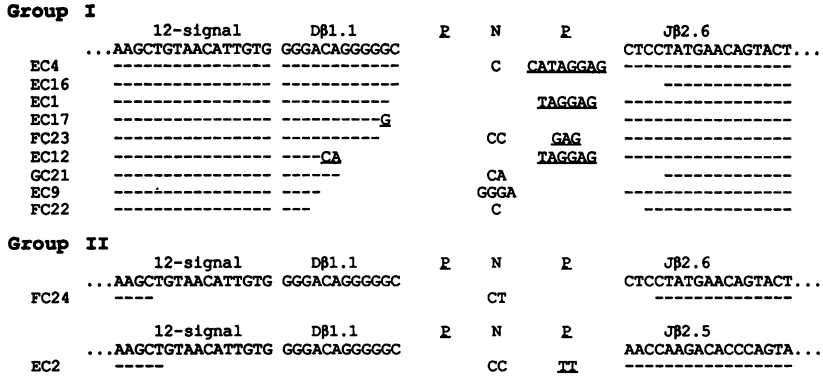

FIG. 8. Dβ1.1-to-Jβ2 rearrangements recovered from thymocytes of adult scid mice. The germ line sequence of the 12/Dβ1.1 signal, Dβ1.1, Jβ2.5, and Jβ2.6 are shown at the top; below are sequences of cloned rearrangements. The format is the same as in the earlier figures. Clones were derived from DNA preparations E, F, and G as described in the legend to Fig. 6.

TABLE 1. Summary of the number of Dδ2-to-Jδ1 rearrangements with normal and abnormal recombination junctions from fetal, neonatal, and adult mice

Class[a]	Junction	No. of rearrangements with junction type in:					
		Wild-type mice				Scid mice	
		Embryo (d16)	Neonate	Adult	Adult (PFGE)[b]	Neonate	Adult
A	12 sig-Dδ2-Jδ1	3	14	4	6	12	4
B	12 s---Dδ2-Jδ1	0	0	5	2	0	0
C	12 s----------Jδ1	0	2	11	8	5	9
D	12 sig-Dδ2-Jδ1	39		22	16	35	24
E	12 si-------2-Jδ1	1		31	46	12	26
(B+C+E)/total		0.02	0.12	0.65	0.72	0.27	0.55
F	12 sig-Dβ1.1-Jβ2		7	11			9
G	12 s-----------Jβ2		1	0			2
G/total			0.12	0.0		0.18	

[a] The classification of recombination junctions is as follows: normal junctions are classes A, D, and F; open-and-shut Dδ2 junctions with loss of 12/Dδ2 signal nucleotides are class B; abnormal Dδ2 deletions with accompanying loss of 12/Dδ2 signal nucleotides are class C; and those with alteration of germ line sequence at the 12/Dδ2 border are class E. Classes A to C, F, and G were ascertained by sequence analysis, and classes D and E were ascertained by the presence or absence of a *Sau*3AI restriction site spanning the 12/Dδ2 border.

[b] PFGE refers to junctions recovered from large-molecular-size DNA (>2,000 kb) fractionated by pulsed-field gel electrophoresis.

fold lower than that of single open-and-shut junctions (34). These findings suggest that one consensus signal may be sufficient for site-specific recognition, cleavage, modification, and rejoining of DNA ends by the VDJ recombinase. The physiological significance of open-and-shut junctions is not clear. They may reflect a corrective response to an "inappropriate interaction" of the recombinase with only one signal or with two different signals, e.g., a cryptic signal and a consensus signal (34).

Open-and-shut junctions also have been detected in retroviral recombination substrates containing two consensus signals (18). Detection was made possible by preventing recombination between the two signals. To prevent recombination, point mutations were introduced into the heptamer of one or both signals prior to infection of wild-type (or scid) recombinase-active lymphoid cell lines with the retroviral vector. Recombinant vectors with one mutated signal were found to contain open-and-shut junctions at the nonmutated signal/coding border in both wild-type and scid cells. Open-and-shut events in scid cells often resulted in large deletions into the coding and signal ends. No open-and-shut junctions were detectable in retroviral substrates containing two mutated signals or a single, nonmutated signal. Thus, in this system, the formation of open-and-shut junctions appears to depend on interaction of the recombinase with two signals.

In the present study, open-and-shut 12/Dδ2 junctions were clearly evident in 19% (7 of 36) of the Dδ2-to-Jδ1 rearrangements from adult wild-type thymocytes (Fig. 2 and 4). This high frequency was unexpected given the low frequency of open-and-shut junctions in recombinant plasmid substrates and the few noted examples of such junctions in rearranged antigen receptor genes (34). The noted examples include one Dδ2-Jδ1 rearrangement with an open-and-shut junction at the 12/Dδ2 border (11) and two cases of PCR-amplified Vα genes with apparent open-and-shut junctions at their Vα/23 borders (50, 51). Two more examples of possible open-and-shut junctions in PCR-amplified products involving Dδ1-to-Dδ2 rearrangements were recently reported by Carroll et al. (8).

All open-and-shut 12/Dδ2 junctions reported here appeared abnormal in that they showed a loss of nucleotides at the 12 signal. The loss of nucleotides at signal ends is not generally seen in V(D)J recombination; signal junctions, open-and-shut junctions, and hybrid junctions all usually contain unmodified signal ends (20, 23, 33–36, 43, 45, 60). Loss of 12/Dδ2 signal nucleotides would prevent the assembly of a functional Vδ-Dδ2-Jδ1 (or Vδ-Dδ1-Dδ2-Jδ1) gene, because the altered 12 signal would be unable to interact with the recombinase (21) and joining of upstream Vδ and/or Dδ1 elements with the Dδ2-Jδ1 complex would be precluded. Thus, rearranged δ alleles with abnormal open-and-shut 12/Dδ2 junctions could be expected to accumulate in the thymocyte population and to be included in the PCR-amplified Dδ2-to-Jδ1 rearrangements. Normal open-and-shut Dδ2 junctions, on the other hand, would serve as substrates for assembly of a functional δ gene and might be too short-lived to detect.

Possible mechanisms for abnormal Dδ2-associated deletions. Different models can be proposed to account for the deletion of Dδ2 and a portion of its 12 signal, as illustrated in Fig. 9. An open-and-shut event may occur at the 12/Dδ2 border before (model A) or after (model B) cleavage at the Dδ2/23 and 12/Jδ1 signals. Two open-and-shut 12/Dδ2 junctions were in fact detected in cloned PCR products from δ loci with an unrearranged Dδ2 element (Fig. 3A). Model C postulates site-specific breaks at the 12/Dδ2 and Dδ2/23 signals and joining of these two signals; the second event would result in joining of the 12/Dδ2 signal to Jδ1. In support of model C is our detection of joined Dδ2 signals (Fig. 3B). Whether such junctions result from a single recombinase-directed event involving the interaction of the two Dδ2 signals is not clear, because the proximity of these signals would pose an energetic and steric barrier to their interaction (22, 34, 59, 61, 62). An alternate possibility involves nearly simultaneous but independent breaks at the 12/Dδ2 and Dδ2/23 signals, as discussed below.

Loss of Dδ2 along with some of its 12 signal nucleotides may not involve an open-and-shut event. In model D (Fig. 9), cleavage at the Dδ2/23 and 12/Jδ1 signals followed by excessive exonuclease-mediated deletion in the 5′ direction yields a hybrid-like junction lacking Dδ2 as well as some 12/Dδ2 signal nucleotides. In model E, formation of a hybrid junction results from cleavage at the 12/Dδ2 and 12/Jδ1 signals and joining of a modified 12/Dδ2 signal to Jδ1. However, this would violate the 12/23 rule (64), which applies to the formation of both standard and nonstandard (e.g., hybrid) junctions (34). Al-

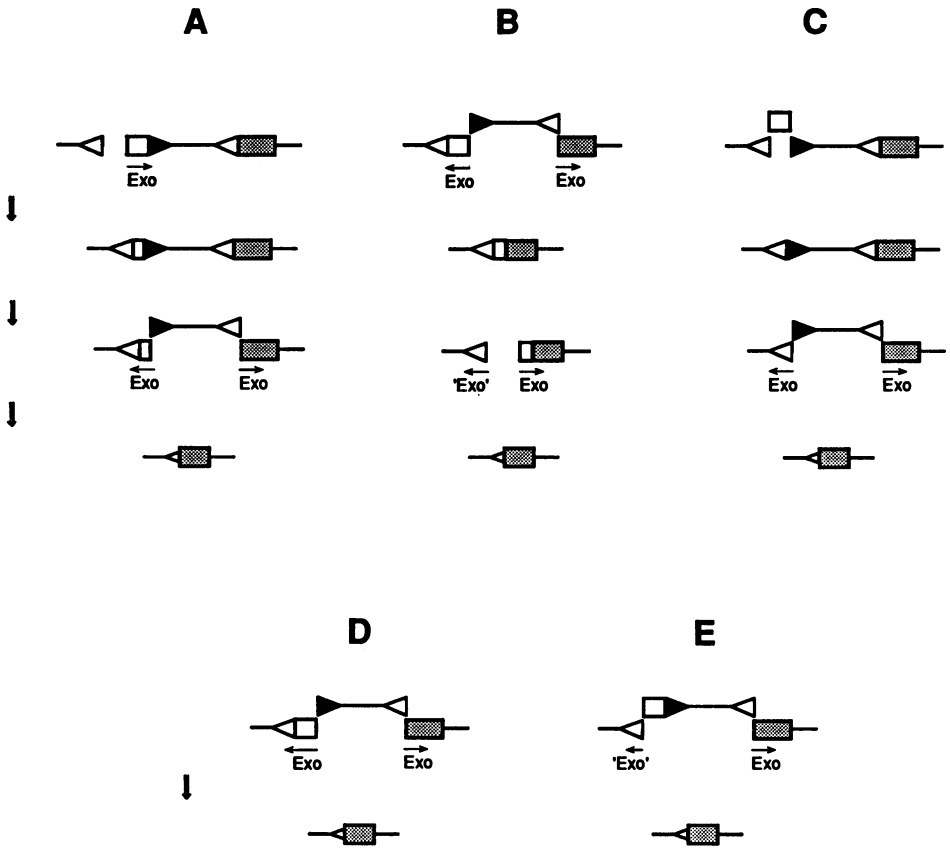

FIG. 9. Models for abnormal Dδ2-associated deletions in rearrangements involving Dδ2 and Jδ1. The elements (and corresponding symbols) are as follows: 12 signal, open triangle; 23 signal, solid triangle; Dδ2, open box; Jδ2, stippled rectangle. Loss of nucleotides at coding ends as a result of exonuclease activity (Exo) is indicated by the arrow. 'Exo' signifies an unusual loss of signal nucleotides. In model A, an open-and-shut junction is formed at the 12/Dδ2 border with loss of Dδ2 (and possibly some signal) nucleotides. The second event involves cleavage at the Dδ2/23 and 12/Jδ1 signals, followed by complete loss of the remaining Dδ2 nucleotides plus some 12/Dδ2 signal nucleotides. The product would resemble a hybrid junction in which there has been an unusual loss of signal nucleotides. Loss of nucleotides at the 12/Dδ2 signal after the first event, together with incomplete deletion of Dδ2 following the second event, could account for the high frequency of abnormal open-and-shut junctions at the 12/Dδ2 border. Model B is similar to model A, except that the order of events is reversed and there is a loss of 12/Dδ2 signal nucleotides during the second event. In model C, the first event involves site-specific breaks at the 12/Dδ2 and Dδ2/23 borders and joining of the 12 and 23 signals to form a signal junction. The second event would involve breaks at the heptamer-heptamer border of the signal junction and at the 12/Jδ1 border, followed by joining of the 12/Dδ2 signal to Jδ1. The 12/Dδ2 signal end would be treated like a coding sequence by the recombinase (33), and the resulting junction could be expected to show a loss of signal nucleotides. Details for models D and E are given in the text.

though this may be sufficient reason to disfavor model E, there are examples of V(D)J rearrangements in which the 12/23 rule has been violated (26, 40, 48). Recombination junctions with missing 12/Dδ2 signal nucleotides might be infrequently generated in model E, but as defective products, they could accumulate and be selectively recovered by our assay.

Occurrence of abnormal Dδ2-associated deletions. Abnormal D-associated deletions were seen at high frequency in Dδ2-to-Jδ1 but not Dβ1.1-to-Jβ2 rearrangements of adult mice. Whether this difference in frequency is peculiar to the elements chosen for comparison or reflects a unique property of the δ locus is not yet clear. At the δ locus, the joining of different coding elements (V to D, D to D, and D to J) occurs concurrently (10), unlike at the β locus, where joining of D to J is followed by that of V to DJ (6, 63) and where D-to-D joining is rare (3, 5, 13, 37). This may reflect differences in the accessibility of each locus to the recombinase. For example, at the β locus, there may be a developmentally controlled gradient of accessibility, starting at a given J region and proceeding 5′ to the D and V regions, whereas at the δ locus, there may be little or no gradient, such that V, D, and J elements are equally (or nearly equally) accessible to the recombinase. This could result in simultaneous interaction of the recombinase with different pairs of signals (e.g., Dδ1/23 and 12/Dδ2 along with Dδ2/23 and 12/Jδ1). Simultaneous interactions could possibly be "sensed" as inappropriate by the recombinase system and could result in the formation of an open-and-shut junction at (i) one (or both) signal/coding border(s) in one of the pairs of interacting signals (e.g., Dδ1/23 and 12/Dδ2), consistent with model A or B in Fig. 9, or (ii) one signal/coding border in each interacting pair (e.g., Dδ1/23 with 12/Dδ2 and Dδ2/23 with 12/Jδ1). Joining of the 12/Dδ2 and Dδ2/23 signals with excision of Dδ2 in case (ii) could account for the signal junction in model C (Fig. 9).

Increased frequency of abnormal Dδ2-associated deletions with age. As indicated above, the δ locus can exist in one of two states: as nonexcised chromosomal DNA (10) or as extrachromosomal circular DNA (45). Thus, one might propose that abnormal Dδ2-associated deletions occur solely on excised DNA and that the proportion of thymocytes ($\alpha\beta^+$ cells) with excised δ loci increases with age. However, if the abnormal deletions were to occur solely on excised DNA, one would not

expect to see them in scid mice. This is because scid thymocytes are developmentally arrested prior to the stage of α gene rearrangement (7, 57). However, scid mice showed an age-dependent increase in frequency of abnormal Dδ2-associated deletions similar to that observed in wild-type mice. Also, in the case of wild-type mice, the use of size-fractionated thymocyte DNA (>2,000 kb), which we presume would be enriched for chromosomal DNA, gave the same result as unfractionated thymocyte DNA.

We are left to consider that most (or all) abnormal Dδ2-associated deletions occur on chromosomal DNA. If this is indeed the case, how does one explain the increased frequency of these deletions with age? Perhaps the simplest explanation is that Dδ2-associated deletions (or cells containing these deletions) accumulate with age because such deletions preclude assembly of a functional Vδ-Dδ2-Jδ1 gene. Another possibility is that the events responsible for the Dδ2-associated deletions occur with higher frequency in thymocytes of adult mice than of fetal or neonatal mice. Alternatively, the responsible events may occur primarily in thymocytes at a particular stage of differentiation, and cells representing this stage may be underrepresented in fetal and neonatal mice. Whether the deletions reside predominantly in $\gamma\delta^+$ or $\alpha\beta^+$ cells or in their precursors is unclear. Also unclear is whether the Dδ2-associated deletions significantly influence the development of $\gamma\delta^+$ or $\alpha\beta^+$ cells. Insights into these issues await the analysis of thymocyte populations representative of different stages of differentiation.

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