

## T-Cell Receptor $\alpha$ Locus V(D)J Recombination By-Products Are Abundant in Thymocytes and Mature T Cells

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**In addition to the assembled coding regions of immunoglobulin and T-cell receptor (TCR) genes, the V(D)J recombination reaction can in principle generate three types of by-products in normal developing lymphocytes: broken DNA molecules that terminate in a recombination signal sequence or a coding region (termed signal or coding end molecules, respectively) and DNA molecules containing fused recombination signal sequences (termed reciprocal products). Using a quantitative Southern blot analysis of the murine TCR  $\alpha$  locus, we demonstrate that substantial amounts of signal end molecules and reciprocal products, but not coding end molecules, exist in thymocytes, while peripheral T cells contain substantial amounts of reciprocal products. At the 5' end of the J $\alpha$  locus, 20% of thymus DNA exists as signal end molecules. An additional 30 to 40% of the TCR  $\alpha/\delta$  locus exists as remarkably stable reciprocal products throughout T-cell development, with the consequence that the TCR C $\delta$  region is substantially retained in  $\alpha\beta$  committed T cells. The disappearance of the broken DNA molecules occurs in the same developmental transition as termination of expression of the recombination activating genes, *RAG-1* and *RAG-2*. These findings raise important questions concerning the mechanism of V(D)J recombination and the maintenance of genome integrity during lymphoid development.**

Vertebrate B- and T-lymphoid cells are able to recognize a large variety of antigens through diverse antigen-specific receptors, the immunoglobulin (Ig) and T-cell receptor (TCR) complexes, respectively. The genes encoding the variable (V), diversity (D), and joining (J) segments of the Ig or TCR molecules are assembled by a somatic rearrangement process termed V(D)J recombination during lymphoid development (1). V(D)J recombination, the only known site-specific recombination reaction in vertebrates, is highly specific to and strictly regulated in immature lymphoid cells. Development of a mature immune system is absolutely dependent on the generation and expression of functional antigen receptors (19, 29, 30, 43).

Some mechanistic aspects of V(D)J recombination (23) as well as certain proteins involved in the reaction have been defined (3, 18, 21, 31, 41, 44), but the precise mechanism and its regulation in vivo remain largely obscure. The reaction is targeted to specific sites in the genome by *cis*-acting elements called recombination signal sequences (RSSs), which consist of conserved heptamer and nonamer motifs separated by random spacer sequences of defined length (48). DNA cleavage occurs at the junction of the individual coding (i.e., V, D, or J) segment and its flanking RSS (Fig. 1) to generate signal ends that are blunt and 5' phosphorylated (42) and coding ends that some evidence suggests terminate in hairpin structures (38, 50). Subsequently, the coding ends, but not the signal ends, typically undergo modification by nucleotide loss and insertion, which alters the protein coding capacity of the original germ line sequences (48). Completion of the recombination process involves the ligation of both the coding and the signal ends (Fig. 1). Genes of the same transcriptional orientation rearrange by deletion to generate extrachromosomal by-products, while those of opposite orientation rearrange by chromosomal inversion (discussed in reference 48).

Predicted intermediates and products of a deletional V(D)J recombination process include double-strand breaks at coding and signal ends and joined coding and joined extrachromosomal signal sequences (referred to as reciprocal products; Fig. 1). Until recently, the quantitative analysis of these products (except the coding joints) was hampered by the technical difficulties of detecting them in vivo. Extrachromosomal circles carrying signal joints of the TCR  $\alpha$  locus (12, 33, 53) and some other loci (32) have been identified, but it was difficult to determine their abundance because of the low and variable yields of extrachromosomal DNA preparations. More recently, signal ends have been detected in the murine thymus for the TCR  $\delta$  locus by Southern blot (39) and for the TCR  $\delta$  (40) and Ig heavy-chain (IgH) loci (42) with ligation-mediated PCR (LMPCR) techniques. Furthermore, significant amounts of TCR  $\delta$  gene coding ends have been identified in the *scid* thymus (38) but not in normal mice (56). Although these studies have provided the first direct evidence for the existence of these intermediates, LMPCR is not quantitative, and the TCR  $\delta$  locus is expected to rearrange in only a small fraction of total thymocytes (37), making the physiological interpretation of these data difficult.

The D, J, and C gene segments of the TCR  $\delta$  locus are located between the TCR  $\alpha$  V and J gene segment clusters (Fig. 2A) (5). Since most (and perhaps all) V-to-J  $\alpha$  rearrangements occur by deletion (see Discussion),  $\alpha$  locus rearrangements lead to deletion of the  $\delta$  locus from the chromosome, which in turn has been assumed to lead to the eventual loss of the  $\delta$  locus from the cell (as a result of either cell division or instability of the extrachromosomal element). We have analyzed the dynamics of V(D)J recombination of the TCR  $\alpha$  locus in adult murine thymus and peripheral T cells with a quantitative multiprobe Southern blot technique. We find that in spite of extensive rearrangement of the TCR  $\alpha$  locus, much of the DNA excised from the chromosome by this process, including the TCR C $\delta$  locus, is retained in thymocytes and even in mature, peripheral T cells. A high proportion of the retained sequences exist on signal end molecules in immature but not in

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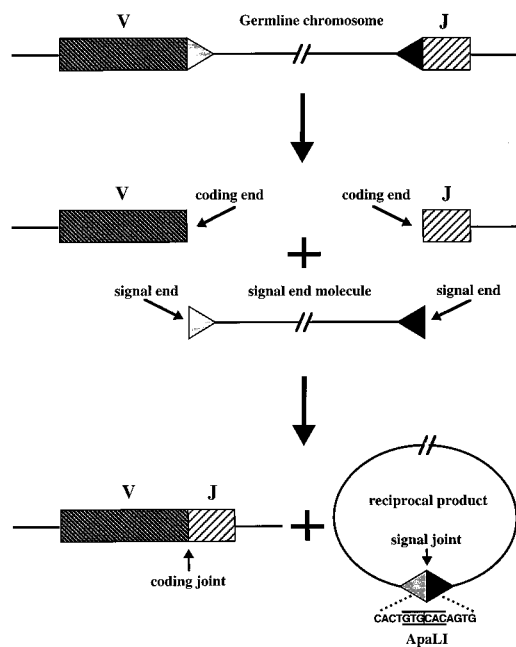


FIG. 1. The substrate, intermediates, and products of V(D)J recombination. (Top) A portion of a chromosome containing a V and J gene segment (rectangles) oriented so as to undergo deletional V(D)J recombination. Flanking each gene segment is an RSS, depicted as a triangle, with the wide end of the triangle the heptamer end and the narrow end of the triangle the nonamer end. The triangles are shaded differently to represent the fact that one RSS has a 12-bp spacer between the heptamer and nonamer elements while the other has a 23-bp spacer. (Middle) The first phase of V(D)J recombination results in cleavage of the DNA between the gene segments and the RSSs to generate a broken chromosome with two coding ends and a signal end molecule with two signal ends. (Bottom) In the second phase of the reaction, the coding ends are processed (not shown) and the break in the chromosome is repaired by the joining of the coding ends to form a coding joint. In addition, the signal ends are joined to form the reciprocal product: an extrachromosomal, circular DNA molecule containing a signal joint. The signal joint is usually a precise fusion of the heptamers of the two RSSs, and this generates a novel site for the enzyme *ApaLI*, as indicated. The signal and coding end molecules and the reciprocal product are referred to here as recombination by-products.

mature thymocytes, with their abrupt decline coinciding with the disappearance of the active V(D)J recombination machinery. Our observations indicate that intermediates of DNA recombination can reach high levels in normal cells and that extrachromosomal sequences can persist for significant lengths of time in mature T cells.

## MATERIALS AND METHODS

**Cell preparations.** Female, 4- to 8-week-old C57BL/6 mice were purchased from Jackson Laboratories. Single-cell suspensions from the thymus and all major lymph nodes were prepared in a tissue grinder. Thymocyte subsets were electronically sorted on FACSTAR Plus (Becton Dickinson) after staining with anti-mouse TCR  $\alpha\beta$  antibody (clone H57-597) conjugated with phycoerythrin (Pharmingen). Purity of TCR  $\alpha\beta^{-lo}$  and TCR  $\alpha\beta^{hi}$  thymocytes was determined by reanalysis with independent staining reactions (see below) and found to be greater than 98 and 95%, respectively. Total peripheral T cells were prepared by panning on anti-murine Ig (Sigma)-coated plates (55). Purified cells were analyzed on a FACSCAN flow cytometer (Becton Dickinson) by staining with one or more of the following monoclonal antibodies: anti-mouse CD3 (clone 29B), anti-mouse CD4 (clone H129.19), and anti-mouse CD8 $\alpha$  (clone 53-6.7) (all from Gibco BRL) and anti-mouse TCR  $\alpha\beta$ , anti-mouse CD45RB, and anti-mouse B220 (clone RA3-6B2) (all from Pharmingen). Live cells were gated with appropriate forward and side scatter settings. Purity of CD3 $^{+}$  peripheral T cells was greater than 95%.

**Nucleic acid preparation and hybridization.** High-molecular-weight DNA was prepared from total homogenized kidney or liver and single-cell suspensions of various T-cell populations with proteinase K digestion and phenol-chloroform

extraction (2). DNA was restriction digested with the indicated enzymes as instructed by the manufacturer (Boehringer Mannheim or New England Biolabs), electrophoresed through 0.7 to 0.8% agarose gel, and transferred to a GeneScreen Plus nylon membrane (Du Pont NEN) on a Posiblot apparatus (Stratagene). Hybridization was carried out in the presence of 50% formamide (2) with random hexamer-primed [ $\alpha$ - $^{32}$ P]dCTP (Amersham)-labeled probes (11). Autoradiography and quantitative analysis were performed by using a PhosphorImager with ImageQuant 3.0 software (Molecular Dynamics). The relative hybridization signal for a given probe was calculated as follows: % relative signal = [(signal for probe of interest in T cell/signal for germ line control in T cell)  $\times$  (signal for germ line control in kidney/signal for probe of interest in kidney)]  $\times$  100.

**Probes.** Probes 5 to 15 and the *RAG-1* probe have been described previously (26). Probe 8a is a 180-bp *BstXI*-*RsaI* fragment of DNA PCR amplified with primers 5'JA50.1 and 3'JA50.1 (Table 1); probe 9a is a *Bam*HI-*SacI* fragment of probe 9 free from the 3' primer sequences. Germ line control probes were Ca I, a 400-bp *Bam*HI-*Hind*III fragment of exons 2 to 4 of the *Ca* gene from a cDNA of the D10 $\alpha$  clone (gift from S. Hong [15]), and Ca II, a 400-bp *XhoI*-*NheI* fragment 3' of the coding sequences of the *Ca* gene from cosmid CaBS2 (provided by D. Sant'Angelo). Probes free from 5' primer sequences used to detect Ja50, Ja49, and Ja42 LMPCR products are probe 8a (see above), probe 10a, a 350-bp *SacI*-*PstI* fragment overlapping 60 bp with sequences upstream from Ja49, and probe 11a, a 370-bp *NsiI*-*Bam*HI fragment of probe 11, respectively. PCR amplifications of the probe fragments carried out in thermocyclers (MJ Research) under standard conditions (2) on C57BL/6 kidney DNA were followed by subcloning into pBKS $^{+}$  II vector.

**LMPCR.** LMPCR detection of broken ends was carried out according to published protocols (42). Briefly, genomic DNA from liver or T-cell samples was treated with RNase A and then alkaline phosphatase (Boehringer Mannheim) or mock treated and then subjected to phenol extraction and ethanol precipitation. The DNA was ligated to a 30- to 50-fold excess of annealed ANC-1 and ANC-2 (Table 1) oligonucleotides with T4 DNA ligase (New England Biolabs) overnight at 15°C. Five to ten percent of the ligation was used in the LMPCRs with one specific 5' primer (for Ja50, 5'JA50.1/2; for Ja49, 5'JA49.1/2; for Ja42 and Ja43, 5' primer of probe 11) in conjunction with the anchor-specific primer APR-1 (Table 1). Standard reactions with either the outer or inner primers were run for 30 cycles. Nested LMPCR was performed with the outer primer for 15 cycles; then 5% of the reaction mixture was transferred into a new PCR with the corresponding inner primer and incubated for another 25 cycles. Control PCRs were carried out specifically for each region with the specific 5' primers listed above and with specific 3' primers, i.e., for Ja50 with the 3' primer of probe 9, for Ja49 with the 3' primer of probe 10, and for Ja42 and Ja43 with the 3' primer of probe 11, for 25 to 27 cycles. One-fourth of the PCR products were separated on 1.2% agarose gels, transferred to a Zetabind nylon membrane (American Bioanalytical), and subjected to Southern hybridization to specific probes (see above). The rest of the outer primer reactions were gel purified and reamplified with the inner primer (or with the same primer for Ja42-43 breaks); specific fragments were subcloned into pBKS $^{+}$  II vector and double strand sequenced on an ABI 373 automated sequencer with a Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems).

**LMPCR cloning of Ja50 coding rearrangements** was carried out on genomic DNA from total thymus samples. DNA was restriction digested with *Pvu*II, phenol extracted and precipitated, and then ligated to annealed ANC-1 and ANC-2 (Table 1) oligonucleotides. Following purification on a microfiltration unit (Millipore), 5% of the ligation was used in an LMPCR with the outer primer 3'JA50.1 (Table 1). Bands of appropriate size, specific to thymus DNA, were gel purified, reamplified with nested primer 3'JA50.2 (Table 1), and subcloned into pBKS $^{+}$  II. Eight independent clones were sequenced as described above.

**ExoV assay.** Genomic DNA from lymphoid and nonlymphoid samples was incubated with or without exonuclease V (ExoV; U.S. Biochemical), at a concentration of 1 U/ $\mu$ g of DNA, in the recommended buffer in the presence of ATP for 1 to 3 h at 37°C. After phenol extraction and ethanol precipitation, the DNA was restriction digested with *EcoRV* or *Bgl*II, Southern blotted, and hybridized to probe 8.

## RESULTS

**Retention of C $\delta$ /Ja locus sequences in  $\alpha\beta$  T cells.** To analyze V(D)J recombination by-products *in vivo*, we chose the murine TCR  $\alpha$  locus as a model system for the following reasons: (i) TCR  $\alpha$  rearrangement takes place in CD4 $^{+}$  CD8 $^{+}$  thymocytes, which represent by far the most numerous, easily accessible lymphoid precursor cells; (ii) the TCR  $\alpha$  locus undergoes extensive (27, 46), repeated rearrangements (34) in CD4 $^{+}$  CD8 $^{+}$  thymocytes, which express high levels of the recombination-activating genes, *RAG-1* and *RAG-2* (4, 35), and (iii) the entire sequence of the murine TCR C $\delta$ /Ja locus is now available (20, 52), facilitating the design and interpretation of complex Southern blot experiments.

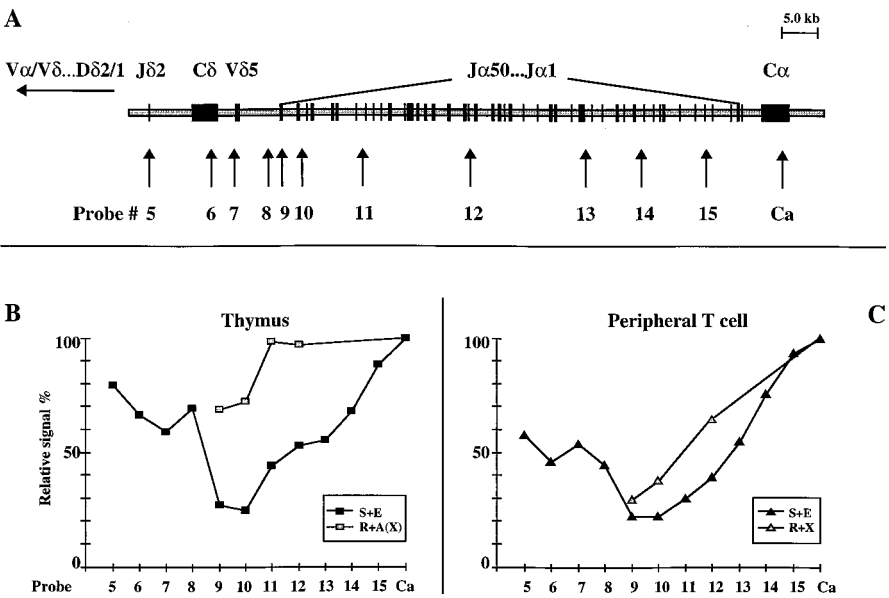


FIG. 2. (A) Schematic map of the murine TCR  $\alpha/\delta$  locus encompassing the region from J $\delta$ 2 through C $\alpha$ , based on the published sequence (20). Closed rectangles represent known genetic elements. Individual exons for C $\delta$  and C $\alpha$  are not shown. The locations of the 11 TCR  $\alpha/\delta$  probes and the control C $\alpha$  probe used in this study are depicted below the map. (B and C) Relative hybridization signal obtained in the germ line-sized band for each of the probes from either thymus (B) or peripheral lymph node T-cell (C) DNA, using different restriction enzyme combinations [*Sph*I-*Eco*RI (S+E) and *Rsa*I-*Ase*I (R+A) or *Rsa*I-*Xba*I (R+X)]. Values are expressed as the percentage of the signal obtained with the relevant probe from the control, nonlymphoid DNA, corrected for DNA loading (see Materials and Methods). The reference probe Ca is set to 100%, assuming that no rearrangement occurs at this probe site. Probes used are indicated by number on the horizontal axis. Values are averages from a number of independent experiments, with the number of determinations with *Sph*I-*Eco*RI for thymus and peripheral T cells, respectively, indicated in parentheses: probe 5 (11, 5); probe 6 (6, 1); probe 7 (7, 2); probe 8 (12, 3); probe 9 (2, 0); probe 10 (17, 6); probe 11 (12, 3); probe 12 (11, 5); probe 13 (9, 3); probe 14 (4, 2); and probe 15 (8, 2). The standard deviations of these values, when more than two determinations were made, in thymus and peripheral T cells, respectively, are as follows: probe 5 (11%, 13%); probe 6 (5%, -); probe 7 (8%, -); probe 8 (12%, 7%); probe 10 (6%, 3%); probe 11 (14%, 7%); probe 12 (13%, 8%); probe 13 (11%, 10%); probe 14 (4%, -); and probe 15 (10%, -) (dashes indicate that no standard deviation could be calculated because there were two or fewer values). Data for *Rsa*I-*Xba*I and *Rsa*I-*Ase*I enzyme combinations were averaged from two independent thymus samples or determined for one lymph node T-cell sample as shown in Fig. 3A. For the thymus, datum points with probes 9 and 11 are derived from *Rsa*I-*Ase*I and datum points with probes 10 and 12 are derived from *Rsa*I-*Xba*I. For peripheral T cells, the value for probe 9 with *Sph*I-*Eco*RI restriction digestion was not independently determined and was extrapolated to be identical to the value for probe 10, as they hybridize to the same fragment (see Fig. 3B), and the hybridization signal for probes 9, 10, and 12 was determined with *Rsa*I-*Xba*I enzyme combination.

We derived 15 probes spanning the TCR  $\alpha/\delta$  locus and used them in various combinations in Southern blot analyses such that each probe hybridized to a single and distinct germ line restriction fragment. DNA from lymphoid (thymus or peripheral T-cell) or nonlymphoid control (liver or kidney) samples were analyzed on the same membrane, and the amount of signal present in the germ line band detected by each probe was quantitated on a PhosphorImager. Variations in DNA loading from lane to lane were corrected for by hybridization with one of two control probes (specific for C $\alpha$  or *RAG-1* sequences) which detect fragments not subject to rearrangement. Results are expressed as the percentage of the signal retained in a particular band from a lymphoid DNA sample,

relative to the signal obtained from the corresponding or reference band with the control DNA sample, corrected for DNA loading (see Materials and Methods). The locations of probes 5 to 15 are shown in Fig. 2A, while the locations of probes 1 to 4 have been reported previously (26).

In such an analysis, there are two mechanisms for the loss of hybridization signal for the germ line restriction fragment recognized by a given probe. If recombination occurs at an RSS within the fragment, the probe site will be placed on a novel, non-germ line-sized restriction fragment. Thus, hybridization signals of germ line restriction fragments spanning RSSs will be reduced in proportion to the extent those particular RSSs participate in rearrangements. Second, if the extrachromo-

TABLE 1. Sequences of oligonucleotides used in LMPCRs

Name	Sequence <sup>a</sup>	Cloning site
ANC-1	GACGCATCGTGTTCGGAAAGCTTCTCGAGGAATTC	<i>Hind</i> III- <i>Eco</i> RI
ANC-2	GAATTCCTCGA	
APR-1	GACGCATCGTGTTCGGAAAGCTTC	<i>Hind</i> III
5'JA50.1	GGTCCACGTCCAGATGCCAACT	
5'JA50.2	GATTAGATCTGGAGAGAGAGGAGTG	<i>Bgl</i> II
5'JA49.1	CCCCAGCACCCAGGACACCTA	
5'JA49.2	GTGGATCCTATCGCTTATTCTATTCT	<i>Bam</i> HI
3'JA50.1	TCGTGGGAAAATTGTAGGTTGT	
3'JA50.2	GCCGGATCCATCTCCCTCCAGCAA	<i>Bam</i> HI

<sup>a</sup> Underlined sequences indicate restriction sites artificially introduced to facilitate subcloning. All sequences are shown from 5' to 3'.



somal reciprocal products of V-to-J $\alpha$  rearrangements are unstable in developing T cells, the corresponding probe sites located on them will be physically lost from the cells.

We first analyzed restriction fragments of an average size of 3 to 6 kb (generated by digestion with *SphI* plus *EcoRI*), which span several RSSs for each J $\alpha$  locus probe (Fig. 2A, probes 9 to 15; for a detailed map of the *SphI* and *EcoRI* sites, see reference 26). Probes 9 and 10 gave a signal of only 20 to 25% of their genomic content (a genomic content of 100% is defined as the signal obtained for the corresponding band from the nonlymphoid, control DNA sample on the same membrane, corrected for DNA loading), while more 3' probes showed a progressive increase in retention of hybridization intensity of the germ line band in total thymus or peripheral T cell DNA (Fig. 2B and C). This is as expected for a locus which rearranges extensively (46) and successively (34). In contrast to what was seen with probes 9 and 10, however, signals obtained with probes 5 to 8 (corresponding to the J $\delta$ 2-C $\delta$  region; Fig. 2A) were retained to up to 60 to 70% of their genomic content in total thymus and up to 40 to 60% in peripheral T-cell DNA (Fig. 2B and C). These results suggest that a significant proportion of V-to-J $\alpha$  recombination reciprocal products are retained in developing and mature T cells.

As noted above, the hybridization signal for a particular germ line restriction fragment can be reduced either by a recombination event involving an RSS within the fragment that alters its size or by physical loss of the fragment from the cell. Since the *SphI-EcoRI* restriction fragments detected by probes 9 to 15 in the J $\alpha$  locus span several RSSs, they are potentially subject to loss by both mechanisms. In contrast, the *SphI-EcoRI* fragments from the C $\delta$  region, detected by probes 5 to 8, span one (probe 7) or no RSSs, and their loss is therefore determined primarily or entirely by their physical elimination. To measure the decrease in hybridization signal exclusively due to the physical loss of the fragments, we used restriction enzymes that place J $\alpha$ -specific probes on short restriction fragments free from RSSs of adjacent J $\alpha$  genes. Use of probes 9, 10, and 12 with *RsaI* plus *XbaI* or probes 9 and 11 with *RsaI* plus *AseI* revealed that even the most upstream probe of the J $\alpha$  locus (probe 9) is retained to 70% of its genomic content, and probes located more downstream (such as 11 and 12) are retained to almost 100% in total thymus DNA samples (Fig. 2B; Southern blot and map shown in Fig. 3). The same trend was observed for peripheral T cells, but with the absolute levels of non-germ line J $\alpha$  locus sequences retained lower than in the thymus (Fig. 2B; Fig. 3A, lane 4), presumably as a result of loss of extrachromosomal DNA during T-cell development and/or division (see Discussion). We conclude that DNA sequences of the entire J $\delta$ 2-C $\delta$ -J $\alpha$  region are retained to >60 to 70% in thymocytes and >30% in peripheral  $\alpha\beta$  T cells. Assuming that the 20 to 25% hybridization signal with probe 9 or 10 in total thymus or peripheral T-cell DNA digested with *SphI* and *EcoRI* (Fig. 2B and C) represents the maximum estimate of intact, unrearranged chromosomal DNA, we calculate that at least 10% (in peripheral T cells) to 40% (in thymus) of the J $\alpha$ 50-J $\alpha$ 45 region sequences (corresponding to probes 9 and 10) are located on non-germ line DNA. By similar reasoning, at least 20% (in peripheral T cells) and 30 to 40% (in thymus) of the J $\delta$ 2-C $\delta$  region is retained as non-germ line DNA. It is important to note that this is not the case in cultured T-cell lines and hybridomas, which have extensive TCR  $\alpha$  gene rearrangements but no detectable TCR  $\delta$  locus sequences (reference 25 and our data for EL-4 [not shown], a thymoma which apparently has both TCR  $\alpha$  alleles rearranged and no TCR  $\delta$  locus sequences retained). This finding suggests that continuously dividing  $\alpha\beta$  T cells do not carry TCR  $\alpha$  and  $\delta$  locus

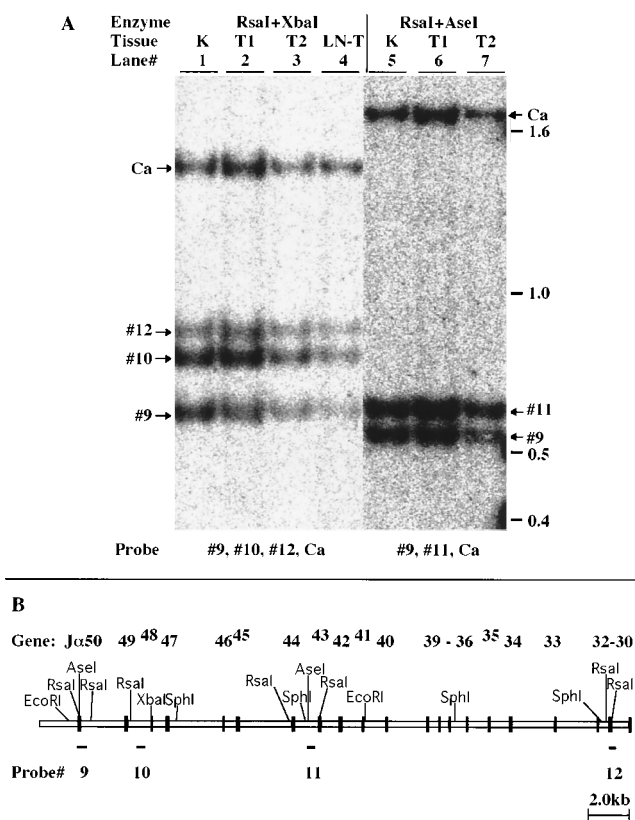
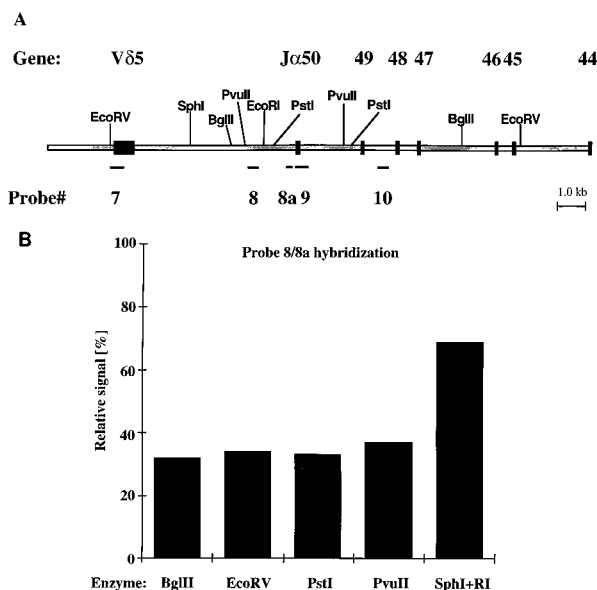


FIG. 3. Retention of TCR  $\alpha/\delta$  locus sequences in  $\alpha\beta$  T cells analyzed on restriction fragments that do not span an RSS. (A) Southern blot analysis of DNA digested with *RsaI* plus *XbaI* (lanes 1 to 4) or *RsaI* plus *AseI* (lanes 5 to 7) and hybridized simultaneously with probes 9, 10, 12, and Ca (lanes 1 to 4) or 9, 11, and Ca (lanes 5 to 7). Samples are DNA from kidney (K) as a germ line control, two independent total adult thymuses (T1 and T2), and peripheral lymph node T cells (LN-T). The germ line band for each probe is indicated with arrows, and molecular size markers in kilobases are shown with dashes. The result of the quantitative analysis is shown in Fig. 2B and C, open symbols. (B) Schematic map of the murine TCR  $\alpha$  locus from J $\alpha$ 50 to J $\alpha$ 30, showing the locations of probes 9 to 12 (small black bars below the map) relative to sites for *AseI*, *EcoRI*, *RsaI*, *SphI*, and *XbaI*. Only the relevant restriction enzyme sites are indicated. Other abbreviations and symbols are as in Fig. 2A.

sequences upstream from their specific J $\alpha$  rearrangements (46) (see Discussion).

In the foregoing analysis using *SphI* and *EcoRI*, probe 8 was retained to 70% of its genomic content in thymus DNA, while the corresponding value for probe 9 was 25% (Fig. 2B). These two probes lie on either side of J $\alpha$ 50, the most upstream J $\alpha$  gene segment (Fig. 2A and 4A), and the combination of *SphI* plus *EcoRI* places probe 8 on a fragment that does not span an RSS and lies entirely upstream of J $\alpha$ 50 (Fig. 4A). We reasoned that if restriction enzymes (e.g., *PvuII* or *PstI*) that placed probe 8 on a fragment spanning J $\alpha$ 50 were used (Fig. 4A; note that these fragments span only J $\alpha$ 50), then the percent retention of the germ line restriction fragment in thymus DNA should drop dramatically if rearrangements to J $\alpha$ 50 are prevalent. When either *PvuII* or *PstI* was used, the germ line restriction fragment detected by probe 8 now was retained to only 30% of its genomic content (Fig. 4B). This result demonstrates that J $\alpha$ 50 is frequently rearranged, with 30 to 40% of all alleles containing a rearrangement to J $\alpha$ 50. In addition, since any rearrangement to J $\alpha$ 50 moves the TCR  $\delta$  locus out of its germ line context, this result proves that the excess hybridization signal of the TCR  $\delta$  locus (as seen with *SphI* plus *EcoRI*

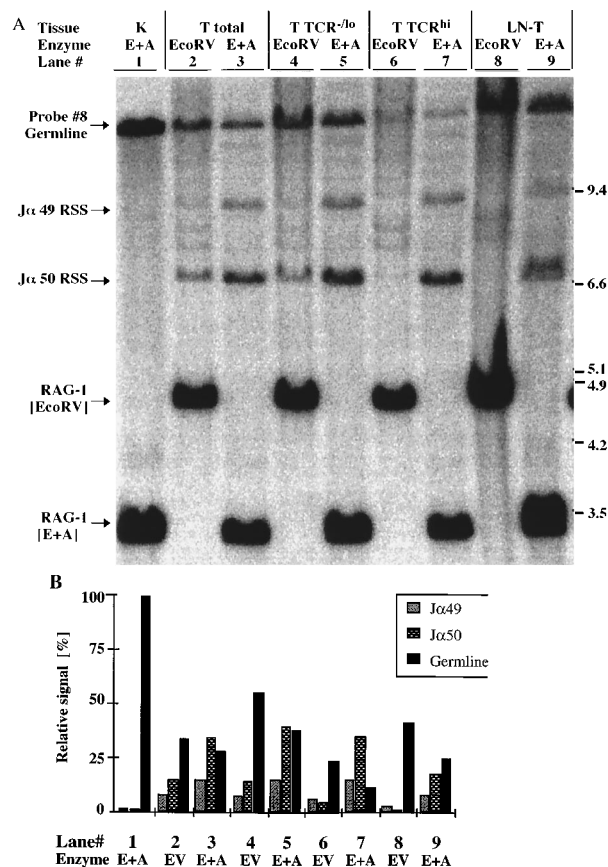


and probes 5 to 8 compared with probes 9 and 10; Fig. 2B and C) is associated with rearranged DNA, most likely in the form of various recombination by-products (see below).

**TCR  $\alpha$  locus signal ends and reciprocal products.** In normal developing thymocytes, non-germ line  $J\alpha$  locus sequences are likely present as either reciprocal products or signal end molecules. Since we observed an especially large discrepancy in hybridization signals between short and long restriction fragments detected with probes 9 and 10 (Fig. 2B), we hypothesized that upstream  $J\alpha$  genes, particularly the first two genes,  $J\alpha 50$  and  $J\alpha 49$ , would serve as the best candidates to quantitate the amount of these recombination by-products present in thymocytes. Broken-end molecules are manifested as novel, non-germ line-sized hybridizing fragments in which one end maps to the same location (the position of the break) with multiple, independent restriction enzymes. Signal joint-containing reciprocal products are also readily identified because the fusion of the conserved heptamers of two RSS elements in a signal joint is virtually always precise, creating a novel site for the restriction enzyme *ApaLI* (Fig. 1). Thus, these molecules yield novel bands of a predictable size by digesting with *ApaLI* and a second enzyme, and furthermore, these *ApaLI*-dependent bands should be identical in size to those obtained by digesting molecules broken at the relevant RSS with the second enzyme alone.

To attempt to identify broken-end molecules terminating in RSSs, we used *EcoRV*, which places probes 8 and 10 on the same germ line restriction fragment and spans the RSSs of

$J\alpha 45$  to  $J\alpha 50$  (Fig. 4A). As discussed above, when probe 8 is placed on a germ line restriction fragment that spans  $J\alpha 50$ , the hybridization intensity of the germ line fragment drops significantly compared with that observed with *SphI-EcoRI* (Fig. 4B). With *EcoRV*, the decrease in the hybridization signal of the germ line fragment is accompanied by the appearance of a series of novel bands migrating typically at smaller than germ line size in both thymus and peripheral T-cell samples (Fig. 5A, lanes 2, 4, 6, and 8). The most prominent of these bands from thymus DNA migrates at the position expected for fragments ending at the  $J\alpha 50$  RSS with a weaker band seen at the position of  $J\alpha 49$  breaks (Fig. 5A, lanes 2 and 4; very weak bands corresponding to  $J\alpha 46$ - $J\alpha 48$  breaks have been observed in some



experiments). Bands of the appropriate size for signal end molecules terminating at the J $\alpha$ 50 RSS were prominent with five additional restriction enzymes (*Pst*I, *Pvu*II, *Hind*III, *Bgl*II, and *Xba*I), while use of *Bgl*II and *Xba*I confirmed the presence of breaks at J $\alpha$ 49 (data not shown). This result demonstrates that total adult thymus DNA contains double-strand DNA breaks prior to the DNA preparation and that with six different restriction enzymes, these breaks map predominantly to the vicinity of J $\alpha$ 50 and, to a lesser extent, J $\alpha$ 49. Comparison of the intensities of these bands to the intensity of the germ line band from kidney DNA (Fig. 5A, lane 1) and correction for DNA loading demonstrate that immature (or total) thymocytes contain J $\alpha$ 50 RSS breaks at levels of 15% of the total genomic DNA content (Fig. 5B), by far the highest proportion of broken DNA molecules so far observed in normal lymphoid precursors.

Combining any of the above-mentioned enzymes with *Apa*LI results in more intense bands of the same size as the J $\alpha$ 50 and J $\alpha$ 49 broken molecules and some reduction in the intensity of the fainter bands migrating at different positions (Fig. 5A, lanes 3, 5, 7, and 9). This result strongly suggests that these faint bands correspond to novel signal joint-containing reciprocal products generated during TCR  $\alpha$  gene rearrangements. As additional physical characterization of the broken-end molecules, we performed limited ATP-dependent micrococcal nuclease (ExoV) digestion followed by *Bgl*II or *Eco*RV restriction digestion (see Materials and Methods). ExoV is a double-stranded-DNA-specific exonuclease that has been used by others to demonstrate broken-end DNA molecules in the context of the TCR  $\delta$  locus (39). We found that the bands corresponding to J $\alpha$ 50 and J $\alpha$ 49 RSS broken ends were sensitive to ExoV treatment, while the reciprocal products identified with *Apa*LI plus either *Bgl*II or *Eco*RV were resistant to the treatment (data not shown).

To investigate the distribution of signal end DNA molecules in T cells at different stages of development, we analyzed DNA from sorted, immature, TCR<sup>-lo</sup> and mature, TCR<sup>hi</sup> thymocytes as well as peripheral T cells with *Eco*RV and *Eco*RV-*Apa*LI digestions. While immature thymocytes exhibited an amount of J $\alpha$ 50 associated signal ends similar to that in total thymus (Fig. 5; compare lanes 2 and 4), mature thymocytes had only a faint signal of the same size, and peripheral T cells gave no detectable signal at the corresponding position (lanes 6 and 8). Use of *Apa*LI demonstrated that reciprocal signal joint-containing molecules are abundant in all of these T-cell subsets (lanes 3, 5, 7, and 9; Fig. 5B for quantitation), suggesting that in mature thymocytes and T cells, only the broken DNA species are eliminated, while reciprocal products are retained at high levels.

To determine if V(D)J recombination intermediates could be identified near other J $\alpha$  genes, we used probe 11 on total thymus DNA digested with *Sph*I or *Hind*III, both of which generate large germ line fragments containing several J $\alpha$  genes (J $\alpha$ 37 to J $\alpha$ 39; Fig. 6B). Both broken-end molecules (Fig. 6A, lanes 2 and 5) and reciprocal signal joint-containing molecules (Fig. 6A, lanes 3 and 6) were found associated with J $\alpha$ 42 and J $\alpha$ 41, but for J $\alpha$ 40 and J $\alpha$ 43, broken-end molecules were below the level of detection and reciprocal products were only very faintly detected (Fig. 6A). No signal was obtained for the more distal J $\alpha$  genes (J $\alpha$ 37 to J $\alpha$ 39). These findings emphasize that extensive rearrangements of the J $\alpha$  genes give rise to high levels of signal end molecules and reciprocal products in the upstream portion of the locus.

For further molecular characterization of the signal end molecules, LMPCR was performed on undigested thymus or control liver DNA with primers specific for J $\alpha$ 50, J $\alpha$ 49, J $\alpha$ 43,

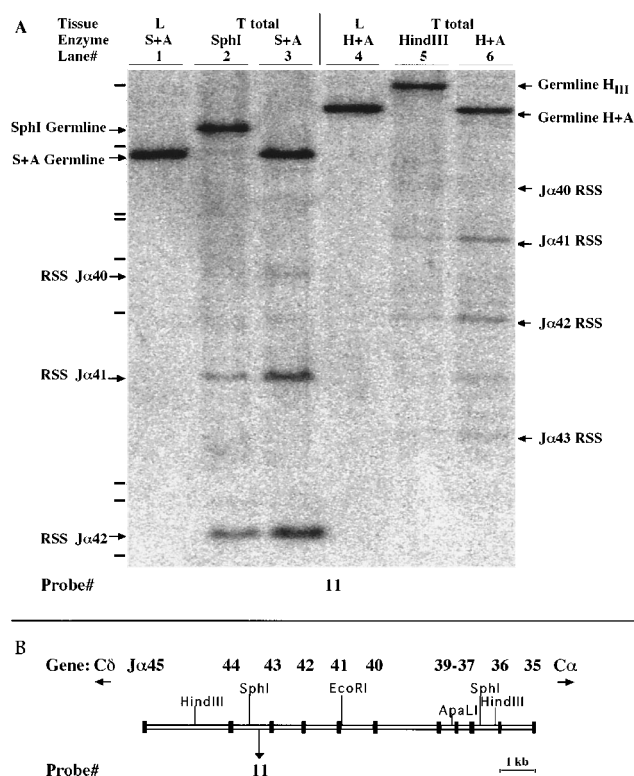


FIG. 6. Detection of reciprocal products and signal end molecules associated with various J $\alpha$  genes in total thymus DNA. (A) Southern blot analysis of DNA digested with *Sph*I, *Sph*I plus *Apa*LI (S+A), *Hind*III, or *Hind*III plus *Apa*LI (H+A) and hybridized with probe 11. Samples are DNA from liver (L) as a germ line control and total adult thymus (T). The positions of various signal end molecules and reciprocal products associated with J $\alpha$ 40 to J $\alpha$ 43 are indicated. Molecular size markers shown on the left with dashes are as follows: 9.4, 6.6, 5.1, 4.9, 4.2, 3.5, 2.0, 1.9, and 1.6 kb. (B) Relevant portion of the map of the murine TCR  $\alpha/\delta$  locus. Symbols are as in Fig. 2A and 3B. Only the restriction enzymes of interest are shown.

and J $\alpha$ 42 (see Materials and Methods). Signal ends were detected in thymus but not in liver DNA (Fig. 7). The double-stranded, blunt-ended molecules are 5' phosphorylated since alkaline phosphatase treatment completely abolished the signal from thymus DNA (Fig. 7), in accordance with recent data obtained by similar methods in studies of the TCR  $\delta$  (40) and the IgH (42) loci. It is interesting that J $\alpha$ 43-associated RSS breaks were not detected even with this sensitive technique whereas J $\alpha$ 42 RSS breaks were readily identified (Fig. 7C), in accordance with the Southern blot analysis (Fig. 6A). The nonamer sequence of the RSS of J $\alpha$ 43 differs in seven positions from the consensus sequence (13), likely resulting in inefficient utilization of this J $\alpha$  gene in TCR  $\alpha$  locus recombination. Sequence analysis of subcloned J $\alpha$ 50-, J $\alpha$ 49-, and J $\alpha$ 42-specific LMPCR products confirmed that the signal breaks terminated precisely at the last nucleotide of the heptamer of the RSS (data not shown).

**TCR  $\alpha$  locus coding ends and coding products.** Since we had identified abundant quantities of J $\alpha$ 50 signal end molecules and reciprocal products, we were interested in determining if J $\alpha$ 50 coding ends could be detected. *Pst*I (Fig. 8A, lanes 1 and 2)- or *Pvu*II (Fig. 8A, lanes 5 and 6)-digested thymus or control kidney DNAs were hybridized with probe 9, since these enzymes generate restriction fragments spanning only the J $\alpha$ 50 gene (Fig. 8B). Double digestion of the control DNA with *Ase*I (Fig. 8A, lane 3) generates a marker band that is only 10 bp



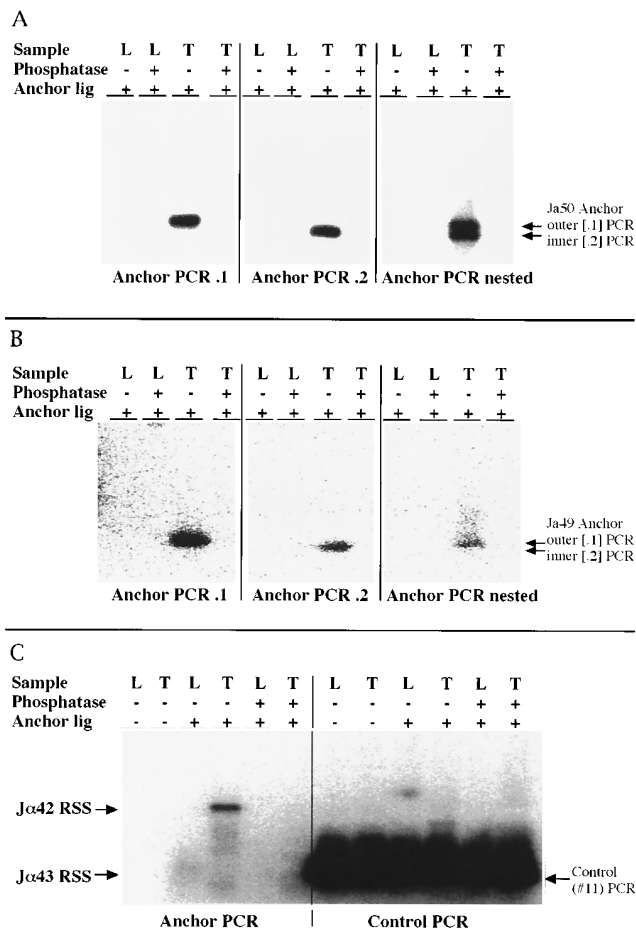


FIG. 7. Demonstration of signal ends associated with  $J\alpha$  genes by LMPCR. Samples are DNA from liver (L) as a germ line, nonbroken DNA control and from total adult thymus (T). The DNA was alkaline phosphatase (+) or mock (-) treated prior to ligation (lig) to the annealed anchor oligonucleotides. After single (.1 and .2 reactions) or nested LMPCR, Southern-blotted samples were hybridized with a specific probe free of the 5' and the anchor primer sequences. The same ligated DNA was used in the control PCRs in panel C. Arrows indicate the expected positions of the various PCR products. (A) Southern hybridization of LMPCR products generated with primers specific for  $J\alpha 50$  RSS breaks with probe 11a. (B) Southern hybridization of LMPCR products generated with primers specific for  $J\alpha 49$  RSS breaks with probe 10a. (C) Southern hybridization of LMPCR products generated with primers specific for  $J\alpha 43$ - $J\alpha 42$  RSS breaks with probe 11a. Samples are as shown for panels A and B except that (i) additional control reactions include liver and thymus DNA mock-ligated samples (-) and (ii) for this particular analysis, only one 5' primer was used and therefore no second or nested PCRs are shown. The control PCR is the same as was used to generate probe 11. Note that  $J\alpha 43$  signal ends cannot be detected despite the smaller expected size of their LMPCR product, whereas  $J\alpha 42$  signal ends are readily amplified.

smaller than the putative coding end molecule (Fig. 8B), which is below the resolution of these Southern blots. Consistent with previous studies on the TCR  $\delta$  locus (39), we could not detect coding ends at  $J\alpha 50$  (gray arrowheads in Fig. 8A, lanes 2 and 6). We do, however, detect novel non-germ line bands with these enzymes (black arrowheads in lanes 2 and 6). That these non-germ line bands correspond to  $J\alpha 50$  coding products is suggested by their disappearance upon double digestion with *AseI*, with coincident appearance of a strong germ line *PstI*-*AseI* band (Fig. 8A; compare lanes 2 and 4), proving that the novel *PstI* and *PvuII* sites are brought in at the 5' end of the fragments.

Some of these non-germ line bands migrate close to the

positions expected for coding end molecules, most clearly seen with *PvuII* (Fig. 8A, lane 6). To determine the molecular nature of this *PvuII* fragment and distinguish it unambiguously from potential coding ends, it was cloned by using LMPCR on *PvuII*-digested thymus DNA (see Materials and Methods), and eight randomly chosen clones were sequenced. This analysis revealed  $J\alpha 50$  sequences joined to members of the  $V\alpha 8$  (four clones) or  $V\alpha 11$  (three clones) gene family, with characteristic modifications at the V-J junctions (data not shown). The eighth clone was derived from the joining of the  $V\delta 4$  gene to the  $J\alpha 50$  gene. Interestingly, all published  $V\alpha 8$  (14) and  $V\alpha 11$  (16) gene sequences, as well as that of  $V\delta 4$  (10), contain a *PvuII* site 35 to 40 bp upstream from their RSSs, and all  $V\alpha 8$  genes have a conserved *PstI* site 18 bp further upstream. This finding helps explain the predominant non-germ line *PvuII* and *PstI* restriction fragments detected close to the position expected for coding end molecules and suggests that conserved *PvuII* or *PstI* sites in the vicinity of other  $V\alpha$  gene families

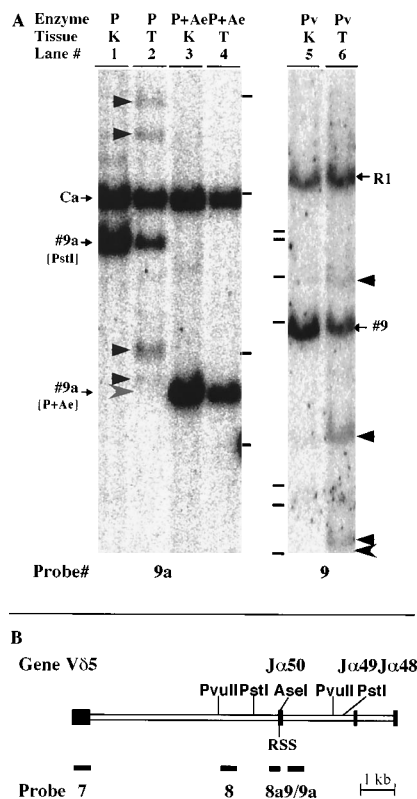


FIG. 8.  $J\alpha 50$  coding ends cannot be detected by Southern blotting of total thymus DNA. (A) Independent Southern blots of DNA digested with restriction enzymes *PstI* (P), *PvuII* (Pv), and *AseI* (Ae) and hybridized with probe 9 or 9a as shown. Samples are DNA from kidney (K) as a germ line control and total adult thymus (T). Arrows identify the positions of the germ line bands. Triangular arrowheads (lanes 2 and 6) indicate non-germ line bands most likely corresponding to  $J\alpha 50$  coding products; forked arrowheads (lanes 2 and 6) indicate the positions where coding end molecules would migrate, as determined (to within 10 bp) by the band obtained with double digestion with *PstI* and *AseI* in lanes 3 and 4. Note that the smallest, distinct, non-germ line *PstI* fragment in lane 2 migrates more slowly than the *PstI*-*AseI* marker band. LMPCR experiments described in the text demonstrate that joining of members of certain  $V\alpha$  families to  $J\alpha 50$  generates probe 9-hybridizing *PvuII* and *PstI* fragments approximately 35 and 55 bp larger, respectively, than the sizes predicted for  $J\alpha 50$  coding end molecules. Dashes indicate molecular size markers of 4.1, 3.0, 2.0, and 1.6 kb (for lanes 1 to 4) and 5.1, 4.9, 4.2, 3.5, 2.0, 1.9, 1.6 kb (for lanes 5 to 6). (B) Relevant portion of the map of the murine TCR  $\alpha/\delta$  locus. Symbols are as in Fig. 2A and 3B. Only the restriction enzymes of interest are shown. *AseI* is located 10 bp 3' from the heptamer of the RSS of  $J\alpha 50$ .

could account for the other discrete, non-germ line fragments seen with *PvuII* or *PstI* (Fig. 8A). Thus, novel, non-germ line bands on TCR  $\alpha$  Southern blots need not result from rearrangement events involving a single V $\alpha$  gene but rather may arise from heterogeneous rearrangements of certain V $\alpha$  gene families with highly conserved coding sequences (see Discussion). While this analysis does not allow us to rule out the presence of low levels of J $\alpha$ 50 coding end molecules, the resolution of our Southern blot is sufficient, with *PstI* digestion (Fig. 8A, lane 2), to distinguish such molecules (forked arrowhead) from the closely migrating band (adjacent triangular arrowhead). No signal is seen at the position expected for J $\alpha$ 50 coding end molecules (Fig. 8A, lane 2), suggesting that if coding end molecules are present, they are below the level of detection of our assay. This point is further emphasized by our inability to detect J $\alpha$ 50 hairpin coding ends by two-dimensional agarose gel electrophoresis and Southern blotting of thymus DNA with probe 9 (data not shown).

## DISCUSSION

Quantitative Southern blot analysis of the murine TCR  $\alpha$  and TCR  $\delta$  loci in  $\alpha\beta$  T cells has revealed a surprising amount of retained DNA sequences of the J $\delta$ 2-C $\delta$ -J $\alpha$  region which were previously thought to be lost from the cell during intrathymic T-cell development. We demonstrate that most of these sequences persist as V(D)J recombination by-products that are in all likelihood extrachromosomal. Approximately 20% of the total genomic DNA of the J $\alpha$  locus exists on signal end molecules in the thymus, whereas the rest of the non-germ line hybridization signal derives from reciprocal products.

**Generation of V(D)J recombination by-products of the TCR  $\alpha$  locus.** In principle, the reciprocal products may exist as either extrachromosomal circles or chromosomal sequences (as a result of either inversional rearrangement or deletion followed by reintegration). Three pieces of evidence support the conclusion that most or all reciprocal products of V-to-J $\alpha$  rearrangement are extrachromosomal: (i) all V $\alpha$  gene segments for which genomic mapping and sequencing data are available are in the same transcriptional orientation as the J $\alpha$  genes (17, 51) and therefore must rearrange by deletion, (ii) the circular by-products predicted for deletional V-to-J $\alpha$  rearrangement have been identified in numerous studies (12, 33, 45, 53), and (iii) established TCR  $\alpha\beta$  T-cell clones or hybridomas do not contain TCR  $\delta$  gene sequences (reference 25 and our results with EL-4 [data not shown]), suggesting that cell division results in the loss of the reciprocal products. In contrast, there is not a single example of inversion in the TCR  $\alpha$  locus. If inversion or reintegration events occur, leading to chromosomal retention of the reciprocal products, they are likely to be rare and make no significant contribution to the total pool of recombination by-products analyzed in this study. Final resolution of this issue awaits the completion of the sequencing of the V $\alpha$  locus (51).

**Do  $\delta$ Rec elements mediate deletion of the TCR  $\delta$  locus in the murine thymus?** It has been suggested that rearrangement of the TCR  $\alpha$  locus is initiated with the deletion of the D-J-C $\delta$  region by V(D)J rearrangements between one or a few  $\delta$ Rec elements and the J $\alpha$ 50 gene (7). Our data are inconsistent with this model in three ways. First, we have demonstrated that the  $\delta$  locus retained in  $\alpha\beta$  lineage thymocytes is predominantly in a VDJ $\delta$  rearranged configuration (26), which is incompatible with  $\delta$ Rec-J $\alpha$  rearrangements given the known location of  $\delta$ Rec elements between the V and D $\delta$  genes (45, 51). Second, if a few genetic elements were responsible for a significant portion of the rearrangements to J $\alpha$ 50, we would expect to

have detected the corresponding reciprocal products as distinct non-germ line bands with probe 8. In accordance with earlier reports (53), however, numerous attempts to detect such bands have failed (Fig. 5A and data not shown), suggesting that at least the J $\alpha$ 50-associated reciprocal products are the result of multiple, diverse recombination events. Third, although probe 9 detected a few predominant, non-germ line bands due to J $\alpha$ 50 rearrangements (Fig. 8A, triangular arrowheads), we proved that at least one band was generated by rearrangements of multiple V $\alpha$  genes instead of a single  $\delta$ Rec element. We conclude therefore that  $\delta$ Rec-J $\alpha$ 50 rearrangement is unlikely to play a significant role in murine T-cell development. Our results with probe 8 and the known capacity of the TCR  $\alpha$  locus to undergo successive rearrangements (34) lead us to favor the idea that many of the reciprocal products that we detect derive from successive rearrangements on persisting circles, leading to the observed very high frequency of rearrangements involving J $\alpha$ 50 (Fig. 4B). The existing data, however, do not rule out that superimposed on this is a tendency toward an ordered progression of chromosomal TCR  $\alpha$  gene rearrangements from 5' to 3' in the J $\alpha$  cluster (47), since there is significantly less rearrangement at the 3' than at the 5' half of the locus (Fig. 2B and C).

**High levels of J $\alpha$  RSS breaks.** It was recently reported that signal end molecules associated with loci undergoing V(D)J recombination can be detected in total thymus DNA by LMPCR (42) or the Southern blot technique (39). The relative abundance of these intermediates was difficult to estimate, however, either because of the nature of the PCR technique or, in the case of Southern blotting, because it remains unclear what fraction of total thymocytes carry out TCR  $\delta$  locus rearrangements. We found numerous signal end molecules in the TCR J $\alpha$  locus in total or immature thymocytes but not in mature thymocytes or peripheral T cells (Fig. 5A). Since approximately 80 to 85% of the total and 95% of the sorted TCR<sup>-lo</sup> thymocyte preparations were CD4<sup>+</sup> CD8<sup>+</sup> thymocytes (see Materials and Methods), we can directly estimate the proportion of signal end molecules in cells which are known to continuously rearrange their TCR  $\alpha$  loci. J $\alpha$ 50-associated breaks account for approximately 15% of the total genomic content, while for other breaks (e.g., J $\alpha$ 49 [Fig. 5A], J $\alpha$ 41 and J $\alpha$ 42 [Fig. 6A], and J $\alpha$ 47 and J $\alpha$ 48 [data not shown]), the value is a few percent for each. Therefore, perhaps 20 to 25% of TCR  $\alpha/\delta$ -containing chromosomes have given rise to a signal end molecule at any one moment, an astonishingly high fraction of the total genomic sequences of the TCR  $\alpha$  locus. These signal end molecules may be relatively short-lived, in which case they must be rapidly regenerated by secondary V(D)J recombination events. Alternatively, signal end molecules might be relatively stable in the presence of the V(D)J recombination machinery. The signal end molecules disappear in the same developmental transition (TCR<sup>-lo</sup> to TCR<sup>hi</sup>; Fig. 5) as does expression of *RAG-1* and *RAG-2* (35), in the absence of cell division or any change in cell cycle status. This finding, combined with recent studies indicating that immature thymocytes contain a substantial excess of Rag-1 and Rag-2 protein molecules ( $10^4$  to  $10^5$  per thymocyte [22]) compared with the number of recombination events performed in the lifetime of a thymocyte, is consistent with the hypothesis that the Rag proteins bind to and stabilize the RSS broken ends.

**Double-strand DNA breaks and cellular development.** Strict regulation of V(D)J recombination is required to prevent the loss of functionally selected, assembled antigen receptor genes; to avoid the formation of potentially autoreactive receptor specificities in mature, functionally competent lymphocytes; and for the maintenance of the integrity of the genome during



lymphoid development. While the first two functions are largely lymphoid cell specific, the last one is presumably accomplished through universal cellular mechanisms operating in any cell capable of responding to DNA damage. It has been shown that Rag-2 protein levels are down-regulated in the S, G<sub>2</sub>, and M phases of the cell cycle (24) and that levels of signal end molecules are also reduced in these phases (42), suggesting that V(D)J recombination may shut down during DNA replication and mitosis. Also, it is assumed that extrachromosomal DNA, unless carrying the necessary *cis* elements for replication, would be diluted out during normal cellular proliferation. Our data demonstrating the abundance of both signal end molecules and reciprocal products (which, as argued above, are primarily extrachromosomal) would, at first glance, be in conflict with these regulatory constraints.

To resolve some of the puzzling aspects of this conflict, one has to consider at least two special characteristics of T-cell development. First, 90% of CD4<sup>+</sup> CD8<sup>+</sup> thymocytes, the cells carrying most if not all the TCR  $\alpha$  signal end molecules in the thymus, are noncycling cells (9), and most peripheral T cells are derived from thymocytes which did not undergo a single cycle of cell division after positive selection (49). The very small amount of D-J $\beta$  locus recombination by-products found in the thymus (32), in conjunction with recent observations indicating that rearrangement of the TCR  $\beta$  locus is completed in early T-cell development prior to the vast expansion of CD4<sup>+</sup> CD8<sup>+</sup> thymocytes (8), suggests that extensive cell division does indeed result in the loss of V(D)J recombination by-products. In addition, *in vivo* gene transfer experiments have demonstrated the stability of extrachromosomal DNA molecules in nonreplicating cells (54). Second, it appears that the V(D)J recombination machinery handles signal ends differently from potential coding ends. We (Fig. 8A) and others (38) have been unable to detect coding ends in normal thymocytes even if the corresponding signal ends are readily identified in the same DNA samples. Our analysis of the J $\alpha$ 50 region, which generates signal ends at 10-fold-higher abundance than those reported for the TCR  $\delta$  locus (38, 39), shows that coding ends are at least 20-fold less frequent than the corresponding signal ends in the same thymocyte populations (estimated from Fig. 5A, lane 2, and 8A, lane 2). These results suggest that coding ends are joined more rapidly than signal ends, despite the fact that coding ends are subject to extensive processing whereas signal ends are not.

Aberrant V(D)J recombination has been speculated to play a role in the generation of common lymphoid malignancies by promoting potentially tumorigenic chromosomal translocations (reference 36 and references therein). A recent report of frequent development of thymomas in  $\gamma$ -irradiated *scid* mice, which generate large numbers of CD4<sup>+</sup> CD8<sup>+</sup> cells (6), raises the possibility that *scid* CD4<sup>+</sup> CD8<sup>+</sup> thymocytes, defective in controlling the rearrangement of the TCR  $\alpha$  locus as a result of the *scid* mutation, are especially sensitive to cellular transformation. Future studies on the generation and abundance of V(D)J recombination by-products in  $\gamma$ -irradiated (6) or TCR  $\beta$ -chain gene transgenic (28) *scid* mice could provide more insight into the regulation of resolution of coding ends and the mechanism of development of thymic tumors.

Our study, the first to demonstrate the quantitative levels of V(D)J recombination by-products in a well-defined immature lymphoid cell population *in vivo*, clearly indicates that these products, even at high levels, are compatible with normal cell development. It remains to be seen, however, to what extent lymphoid cell-specific factors or rather general cellular mechanisms play a role in allowing the coexistence of cellular differentiation and extrachromosomal DNA.

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