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A δ T-Cell Receptor Deleting Element Transgenic Reporter Construct Is Rearranged in αβ but not γδ T-Cell Lineages

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T cells can be divided into two groups on the basis of the expression of either αβ or γδ T-cell receptors (TCRs). Because the TCR δ chain locus lies within the larger TCR α chain locus, control of the utilization of these two receptors is important in T-cell development, specifically for determination of T-cell type: rearrangement of the α locus results in deletion of the δ coding segments and commitment to the αβ lineage. In the developing thymus, a relative site-specific recombination occurs by which the TCR δ chain gene segments are deleted. This deletion removes all Dδ, Jδ, and Cδ genes and occurs on both alleles. This δ deletional mechanism is evolutionarily conserved between mice and humans. Transgenic mice which contain the human δ deleting elements and as much internal TCR δ chain coding sequence as possible without allowing the formation of a complete δ chain gene were developed. Several transgenic lines showing recombinations between deleting elements within the transgene were developed. These lines demonstrate that utilization of the δ deleting elements occurs in αβ T cells of the spleen and thymus. These recombinations are rare in the γδ population, indicating that the machinery for utilization of δ deleting elements is functional in αβ T cells but absent in γδ T cells. Furthermore, a discrete population of early thymocytes containing δ deleting element recombinations but not Vα-to-Jα rearrangements has been identified. These data are consistent with a model in which δ deletion contributes to the implementation of a signal by which the TCR α chain locus is rearranged and expressed and thus becomes an αβ T cell.

Peripheral T cells can be divided into two groups on the basis of the heterodimeric antigen receptor displayed on the surface of the cell. Most mature T cells have α and β chains paired with CD3, while T-cell receptor (TCR) γ and δ chains are associated with CD3 on quite different T cells (1, 5, 10, 21, 32, 38). These two types of T cells have different tissue distributions and appear to have different functions (2, 4, 43).

While heavy and light immunoglobulin chains, as well as TCR β and γ chains, have distinct chromosomal localizations (30, 31), the TCR δ chain locus is contained within the much larger TCR α chain locus on chromosome 14 in both mice and humans (9, 22, 24, 36, 45, 49). This unique organization of TCR loci poses interesting questions concerning the use of these distinct receptor chains. TCR α and δ chains never appear on the same cell, implying exclusivity. Aside from a few variable (V) regions which can be found in α or δ chains, there is no mixing of the elements between these genes (8, 15). There must exist mechanisms that prevent incorporation of internal δ chain segments in α chains and that inhibit TCR α chain rearrangement in γδ T cells.

A novel rearrangement observed in early thymocytes might implement the choice between becoming an αβ T cell and becoming a γδ T cell. This step involves deletion of the δ locus in cells destined to become αβ-bearing T cells. Two elements flanking the D, J, and Cδ segments preferentially recombine at very high frequency in the DNA of polyclonal human thymocytes, resulting in the loss of internal δ coding segments (12). The upstream δ deleting element, δREC, lies ≈120 kb upstream of Cδ; has no V, D, Jα, or δ homology; has no obvious open reading frame; and appears to be an isolated heptamer-spacer-nonamer (h-s-n) recombinatorial signal. The 3′ δ deleting element, ψJα, has lost consensus amino acids contained in all functional Jα and Jδ segments but retains its recombinase recognizing the h-s-n motif. The δREC-to-ψJα (δREC-ψJα) recombination is a chromosomal intermediate rearrangement that is lost on both alleles in mature peripheral αβ T cells (12, 23).

To assess the importance of this event, we looked at the evolutionary conservation of the deleting elements. We found that both elements were conserved between mice and humans in sequence and localization (26). These elements, δREC and ψJα, flank the δ locus in the mouse, undergo rearrangement with each other in the murine thymus, and are lost in mature αβ T cells. One difference noted between the two systems related to the frequency of specific δREC-ψJα recombinations. The mouse thymus contained fewer alleles undergoing δREC-ψJα recombinations than the human thymus, with the murine δREC-ψJα recombination barely detectable by Southern blot analysis (26). In this study, we provide evidence that the mouse uses numerous 3′ acceptor sites instead of predominantly one site.

At issue is whether these δREC-ψJα and δREC-Jα rearrangements are intermediate prospective δ deletion events that eliminate the possibility of making a TCR δ chain. The δ deleting elements would implement a signal to rearrange and express TCR α chains and thus select the αβ lineage. This model necessitates that δ deleting element recombinations be restricted to the αβ T-cell lineage and that they occur prior to Vα-Jα joining. While δREC-ψJα rearrangements are found within chromosomal DNA, it has been difficult to determine if δ deletion rearrangements occur as an antecedent to Vα-Jα

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joining in an individual cell. To assess whether δREC-ψJα recombinations were restricted to the αβ lineage versus the γδ lineage, we generated mice bearing a δREC-ψJα "passenger" transgene. Since the δREC-ψJα transgene was separate from the α-δ locus, its rearrangement could now be monitored in mature γδ and αβ T cells. We report evidence that δ deletion rearrangements are frequent and are restricted to the αβ T-cell lineage.

Several attempts have been made to define discrete stages of T-cell development delineated by the cell surface markers expressed (19, 20, 40–42, 47). Most of these models have used antibodies specific for cell surface markers, alone or in combination, to sort the different populations and have used thymic repopulation studies to describe subsequent stages of T-cell development. The most commonly used markers for this type of analysis have been the interleukin 2 (IL-2) receptor (CD25) and Pgp-1 (CD44). Recently, the status of TCR genes in these populations has been assessed (19, 55). The γ and β chains appear to contain the earliest detectable recombinations, followed by the α chain. The δ chain also appears to be rearranged early, but the exact relationship between TCR δ rearrangement and those of the other TCR chains has not been defined.

The rationale behind this study involved use of antibodies specific for cell surface molecules to isolate defined stages in the maturation of T cells and to assess the status of TCRs and δ deleting elements. Another example of such a molecule, besides CD25 and CD44, is the cell surface marker 11B5, first isolated and described by J. Cain in the laboratory of Max Cooper. This molecule stains thymocytes early in development and correlates with the expression of CD25 and CD44 molecules (11B5 subdivides the CD44− CD25+ population, with $11B5^+$ representing the more immature T-cell stage [6, 19]) on the surface of developing thymocytes. With this molecule, we present data about the early stages of thymocyte differentiation that suggest that δ deletion occurs prior to Vα-Jα joining.

MATERIALS AND METHODS

Southern blotting. DNA was isolated from tissue culture cells as described previously (46). DNA from mouse tails and organs was isolated as described previously (37). High-molecular-weight DNA was digested, electrophoresed, transferred, and hybridized as described previously (26).

PCR. PCR was carried out essentially as described previously (46) with 0.5 μg of genomic DNA per reaction tube. The primers for all PCR mixtures are listed in Table 1. The parameters for amplification of murine δREC-$J\alpha_x$ were 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s for 30 cycles. The parameters for amplification with all TCR V to J primers were 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s for 30 cycles. Probes for each TCR chain were made by PCR with V segment-specific primers internal to the primer used in PCR mixtures for V-J recombination. PCR product blots, representing 20% of the reaction volume, were electrophoresed in 1% agarose gels, transferred, and hybridized as described above for high-molecular-weight DNA.

Quantitative PCR. Specific δREC recombinants were quantitated by a modification of the procedure described in reference 25. The competitor pQPCR. δREC1 (see Fig. 6) was constructed by ligation of the 5′ and 3′ respective oligonucleotide templates into the general cloning vector pQPCR1 as described previously (25). After sequence confirmation, pQPCR.δREC1 was linearized with *Xho*I, gel purified, and quantitated by optical density at 260 nm. Appropriate dilutions were made in H_2O with 10 μg of MS-2 phage RNA (Boehringer Mannheim, Indianapolis, Ind.) per ml. These dilutions were used directly in PCR mixtures. Quantitative PCR was performed with 10-μl reaction mixtures with 0.5 to 1 μg of genomic DNA (αβ or γδ T-cell DNA) in 0.5-ml Eppendorf tubes for 30 to 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s. After PCR amplification, enzyme immunoassay detection was performed as described previously (25). Hybridization oligonucleotides were 5′-TCAAGGCTTATGGAA AAAGACATTA-3′ for the "stuffer" and 5′-GATCCTCAAGGGTCGAGACT GTCATAC-3′ for transgenic δREC recombinant detection. Note that the spacer and poly(A)+ tail are not utilized in DNA quantitative PCR but are exploited when an RNA competitor is used (25).

Transgenic mouse construction. The predominantly human transgenic construct was built in five steps. Step one consisted of subcloning of the 6.0-kb *Cla*I-to-*Bam*HI fragment containing human ψJα and $J\alpha_{56–60}$ from phage 111a (24) into the plasmid vector Bluescript (Stratagene, San Diego, Calif.). Step 2 involved addition of the 8.8-kb *Bam*HI fragment containing all coding segments of murine Cα plus the TCR α chain enhancer, Eα (kindly supplied by Dennis Loh, Washington University, St. Louis, Mo.). Once that was accomplished, the newly generated 14.8-kb fragment of step 2 was cut on the 5′ end with *Sal*I, linkers were ligated onto the ends to recreate the *Sal*I site, and a *Not*I site flanking the *Sal*I site was added. The ligation reaction was then digested with *Not*I, and the 14.8-kb fragment was separated from the Bluescript backbone. The 14.8-kb *Not*I fragment was subcloned into the cosmid vector Supercos I (Stratagene), which had the vector *Cla*I site destroyed by Klenow fill in. Step 4 entailed addition of the 16-kb *Sal*I-to-*Cla*I fragment spanning Jδ3 through TEA (containing Cδ) from phage P3.1 (24). Step 5 entailed addition of the δREC 1.9-kb *Sal*I-to-*Xho*I fragment from phage 5AB (24) into the *Sal*I site of step 4. In each of the steps, where appropriate, the correct insert size and orientation were determined by restriction digestion and Southern blotting. Transgenic founder lines were created by injection of construct DNA into day 1-fertilized embryos as previously described (37).

Isolation and expansion of γδ T cells. γδ T cells were isolated from the thymuses or spleens of four animals at a time. Single-cell suspensions of thymocytes or splenocytes in Dulbecco's modified Eagle's medium were isolated on petri dishes (100 by 15 mm in diameter) (Fisher Scientific) that had been coated with the anti-δTCR antibody 403A10 (kindly supplied by Osami Kanagawa). Plates were washed to remove the remaining αβ T cells and immature T cells. Nonadherent cells, stripped of γδ T cells, were collected, the DNA was prepared, and then the cells were used as sources of polyclonal T-cell DNA. Dulbecco's modified Eagle's medium with 10% fetal calf serum and 50 IU of recombinant

TABLE 1. Primers used in this study

Primer	Primer sequence	Reference
Murine		
$J\alpha_{11}$	5′-GACCCTATTACTCACATACT-3′	54
$J\alpha_{19}$	5′-ATCAAGTCTTTCTTGCTTGT-3′	54
$J\alpha_{58}$	5′-CCAGGAACAGGAGAAGACAC-3′	54
$J\alpha_{65}$	5′-ACTTACTTGGAATGACAGTC-3′	54
$J\alpha_{80}$	5′-CAATGTCCTCTATATAGTTC-3′	54
ψJα	5′-GGCCACTGTTAACTATGTCT-3′	26
$V\alpha_3$	5′-CACCTTATCTGTTCTGGTAT-3′	51
$V\beta_8$	5′-CAAGGTGACAGTAACAGGAG-3′	3
$J\beta_{1.5}$	5′-GAACAGAGAGTCGAGTC-3′	18
$V\gamma_{1.2}$	5′-CTTGGGCAGCTGGAGCAAAC-3′	17
$J\gamma_2$	5′-CGGAGGGAATTACTATGAGC-3′	17
$V\delta_4$	5′-GTGTCTCAGCCTCAGAAGAA-3′	15
$J\delta_2$	5′-ACAAAGAGCTCTATGCCAGT-3′	15
δREC	5′-CCATTTGGAGAATTGATGCC-3′	26
Internal $V\alpha_3$ probe		
Sense	5′-CAGCTGAGATGCAAGTATTC-3′	51
Antisense	5′-CAGAAGTACACAGCCCA-3′	51
Internal $V\beta_8$ probe		
Sense	5′-GGTGACATTGAGCTGT-3′	3
Antisense	5′-CTGGCACAGAAGTACA-3′	3
Internal $V\gamma_{1.2}$ probe		
Sense	5′-GTGCAAATATCCTGTATAGTT-3′	17
Antisense	5′-ACAGTAGTAGGTGGCTTCAT-3′	17
Internal $V\delta_4$ probe		
Sense	5′-GCAGGTGGCAGAATC-3′	15
Antisense	5′-ATACGTCGCAGCATC-3′	15
Human		
δREC	5′-CCCTACTTCGTATGTTAGAC-3′	24
ψJα	5′-GTTCCTGTTTGTTAAGGCAC-3′	24
$J\alpha_{57}$	5′-CAGGTCCAGGAGAAATTTGGAAGC-3′	28
$J\alpha_{58}$	5′-ACTGTGAGCTGTGTTCCTTC-3′	28
$J\alpha_{59}$	5′-TTGCGTCCCCATTCCAAATG-3′	28
$J\alpha_{60}$	5′-CAATTAACTCAGTCCCCTTC-3′	28
Internal δREC probe		
Sense	5′-GAATTAATACAGCCTTGACT-3′	24
Antisense	5′-GGTGCCTATGCATCACCGTG-3′	24

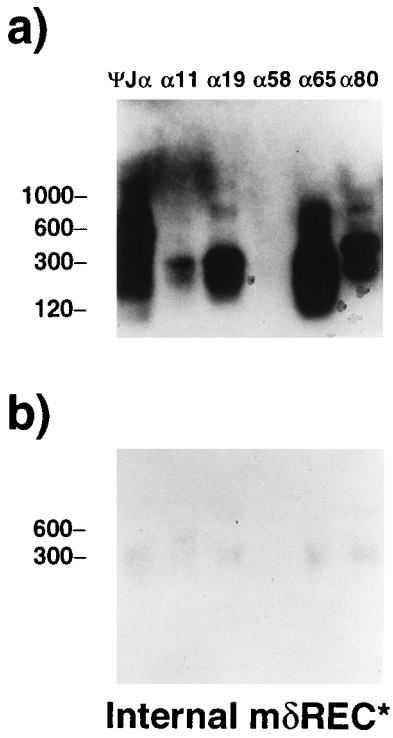


FIG. 1. Presence of δREC-Jα recombinants in murine thymus and spleen. Results of a Southern blot hybridized with a radiolabeled murine δREC probe of PCR products obtained with δREC and various Jα primers in neonatal day 1 thymus (a) and neonatal day 3 spleen (b) are shown. The PCR product of δREC and ψJα primers was used as a reference control. Primer pairs used in day 1 thymus and day 3 spleen are identical. Molecular sizes are given to the left in base pairs.

IL-2 per ml was added to the panned γδ T cells, and the plates were incubated for 7 days. Cells were lysed on the plates with 5 ml of 50 mM Tris (pH 8.0)–100 mM EDTA–100 mM NaCl–1% sodium dodecyl sulfate (SDS). Proteinase K (2 mg) was added, and the solution was incubated at 52°C overnight. Extraction, precipitation, and analysis were performed as described above.

DNA sequencing. Double-stranded sequencing of subcloned δREC recombinants in pBluescript was done with T7 polymerase (U.S. Biochemical Corp., Cleveland, Ohio) and dideoxy chain termination (44).

Immunofluorescence cell sorting. Thymocytes from 10 DBA/2 mice or 10 transgenic mice (C3HxC57/B6) (3 to 6 weeks old) suspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin and stained with fluorescein isothiocyanate-conjugated CD8 (CD8-FITC) (Becton Dickinson, Mountain View, Calif.) were removed by being panned on petri dishes coated with goat anti-rat immunoglobulin (Southern Biotechnology Assoc., Birmingham, Ala.). Nonadherent cells were stained with CD3-FITC and CD4-FITC antibodies (Pharmingen, San Diego, Calif.), followed by biotinylated 11B5 antibody and streptavidin-phycoerythrin (PE). Cells which lack expression of CD3, CD4, and CD8 antigens were isolated on the basis of 11B5 antigen expression by being sorted on a FACSTAR IV PLUS (Becton Dickinson). Negative and positive populations were collected in fetal bovine serum. Typical yields were 2×10^5 to 5×10^5 cells. Adherent $CD8^+$ T cells were lysed on the plates as described above for γδ T cells, and the DNA was extracted and used as a source of purified $CD8^+$ T-cell DNA.

Extraction of DNA for PCR. Sorted cells were spun in Eppendorf tubes at 1 to $2{,}000 \times g$. Nuclei were prepared by 1% Triton lysis, centrifuged, and solubilized in 1% SDS. DNA was cleaned by overnight digestion with proteinase K and phenol extraction. Precipitated DNA was resuspended in 10 μl of H_2O for every 100,000 starting cells, and 1 μl was used for each PCR.

RESULTS

Murine δREC uses multiple 3′ acceptor sites. PCR analysis of normal mouse thymus DNA revealed that δREC uses multiple Jα segments as 3′ deleting elements (Fig. 1a). The sequences of five Jα regions (no. 11, 19, 58, 65, and 80 according to the nomenclature of Winoto et al. [54], which correspond to

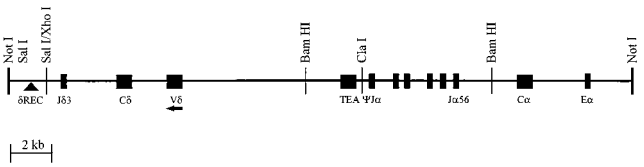


FIG. 2. Schematic representation of the δ deletion transgene. Vδ, δ chain variable segment; Jδ, δ chain joining segment; Jα, α chain joining segment; Cδ, δ chain constant region; Cα, α chain constant region; Eα, α chain enhancer.

Jα regions 25, 33, 28, 41, and 37, respectively, of Koop et al. [28]) which reside between 10 and 50 kb downstream of Cδ were chosen at random for PCR analysis. Of the five Jα sequences chosen, four clearly show rearrangements to δREC by PCR product Southern blotting. The expected size for these murine δREC-Jα PCR products is 300 bp. The range of sizes seen in Fig. 1 could represent heteroduplex formation of DNA products (56) (described below) or possibly δREC rearrangements into adjacent Jα segments, thus giving a larger than expected size (for example, $J\alpha_{65}$ is ≈100 bp from the next Jα segment [29]). Products from two of these δREC-Jα recombinants were subcloned, and their sequences were determined to prove their authenticity (23). A chromosomal intermediate recombination such as these δREC-Jα joints would be deleted upon subsequent Vα-Jα joining when forming functional TCR α chains. As expected, there is a marked diminution in specific δREC-Jα intermediate recombinations, which are only barely detectable in the mature αβ T cells of the spleen (Fig. 1b, blots a and b hybridized concurrently).

Construction of the δREC-ψJα passenger transgene. Results from previous studies and the δREC-Jα PCR data in Fig. 1 indicated that mice use 5′ δREC and 3′ ψJα or Jα deleting elements as do humans. Moreover, these murine δREC-Jα rearrangements would not be stable chromosomal recombinations but would be eliminated by any subsequent Vα-Jα rearrangement when generating αβ T cells. Consequently, we generated a transgenic construct of the human δ locus with the ability to undergo δ deletion to serve as an independent reporter of δ deletion rearrangements (Fig. 2). A predominantly human transgene was chosen because the high degree of sequence homology and similar usage pattern (26) suggested that human sequences would be functional in the mouse, and thus detection of transgenes and transgenic recombinations by PCR and Southern blotting would be facilitated. Because the factors dictating δ deletion are poorly understood, a considerable expanse of DNA surrounding the deleting elements was included. This construct has the 5′ deleting element, δREC, with ≈1 kb of flanking DNA both upstream and downstream.

The region surrounding the 3′ δ deleting element, ψJα, was more extensive. If suppression of TCR α recombinations occurs in the γδ T-cell lineage, a likely location for putative controlling elements would be between $J\delta_3$ and the 5′-most Jα segment. $J\delta_3$ and four human Jα segments ($J\alpha_{56-60}$ [28]) were included in this stretch of DNA. Caution was taken to avoid inclusion of enough TCR δ chain coding elements to form a complete receptor, with the possibility of altering T-cell physiology (the construct had no Dδ segments). Because TEA may be important in directing recombinations to ψJα in the human TCR α-δ locus, it was included in its normal location. The TEA splice partner Cα was added in case the TEA-Cα transcript has an important, unknown function in the process. At the time this construct was generated, the TCR δ chain enhancer had not been described. In case an enhancer element was necessary for efficient δ deletion and because the TCR α enhancer (Eα)

TABLE 2. Transgene summary: rearrangement of δREC to ψJα

Founder	Result by:	
	PCR	Southern blotting
2	−	Scrambled
9	+	Yes
14	+	Yes
17	+	Yes
22	+	Yes
24	+	Yes
36	ND[a]	ND

[a] ND, not determined.

may have been important in the formation of TEA-Cα transcripts, murine Eα was also included. The resultant transgene represents a passenger reporter for the occurrence of δREC recombinations, which would not be subsequently lost on the joining of Vα to Jα.

A total of seven founder lines were obtained, and these are outlined in Table 2. In founder line 2, the DNA construct was scrambled on insertion (23), and founder line 36 was mosaic. Neither of these lines was further analyzed. The five remaining lines all contain an estimated copy number of 10 to 20 copies and had the predicted restriction pattern with multiple restriction enzymes when assessed by Southern blotting (23).

All transgenic lines contain evidence for δREC-ψJα recombination. As seen in Fig. 3 and Table 2, all five remaining transgenic lines contained δREC-ψJα recombinations in T-cell populations by Southern and PCR analyses, most notably in polyclonal thymus. The expected δREC-ψJα joint is a 3.6-kb

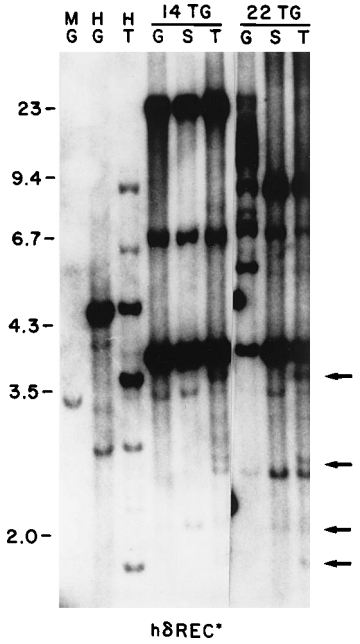


FIG. 3. Recombinants of transgenic δREC in tissues of transgenic lines 14 and 22. Southern blot of *Hin*dIII-digested DNA probed with the 1.7-kb *Sac*I-*Eco*RI human δREC probe (12). The top arrow notes the expected human δREC-ψJα recombination, and the other arrows note alternate δREC recombinations. The bands between 4.5 and 6.5 kb are inconsistent and represent partial digestion artifacts. MG, mouse placenta as germ line control; HG, human placenta as germ line control; HT, human thymus; G, transgenic tail as germ line control; S, transgenic polyclonal spleen; T, transgenic polyclonal thymus. Molecular sizes are given to the left in kilobases.

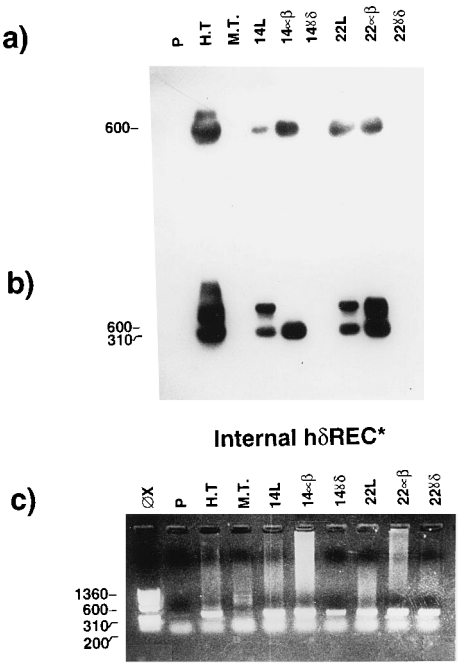


FIG. 4. PCR detection of transgenic δREC-ψJα and δREC-Jα_{57} rearrangement in αβ and γδ T cells from transgenic lines 14 and 22. Results from a Southern blot hybridized with a radiolabeled internal human δREC probe of PCR products obtained with δREC, ψJα, and Jα_{57} primers are shown. (a) δREC-ψJα recombinants. (b) δREC-Jα_{57} recombinants. (c) PCR control for transgenic human δREC. Note that the primers for human δREC in panel c detect germ line and rearranged copies of human δREC. P, primers only; H.T., human thymus DNA; M.T., nontransgenic murine thymus DNA; 14L, transgenic line 14 liver DNA; 14αβ, transgenic line 14 polyclonal thymus DNA with γδ T-cells depleted; 14γδ, transgenic line 14 polyclonal thymic γδ T cells; 22L, transgenic line 22 liver DNA; 22αβ, transgenic line 22 polyclonal thymus DNA with γδ T cells depleted; 22γδ, transgenic line 22 polyclonal thymic γδ T cells. Molecular sizes are given to the left in base pairs.

*Hin*dIII band as outlined in the human thymus DNA and is seen as a submolar rearranged band in the thymus DNA of the 14 and 22 transgenic lines (top arrow in Fig. 3). All transgenic lines showed similar rearrangement patterns (23); therefore, only lines 14 and 22 were analyzed further. Additional recombinations between δREC and other unknown elements can also be detected in thymus and spleen when probed with human δREC (arrows in Fig. 3). Note the bands detected between 4.5 and 6.5 kb. These bands are an inconsistent finding from liver, tail, polyclonal thymus, and polyclonal spleen DNA and likely represent partial digestion artifacts (23).

δREC is rearranged in the αβ but not γδ T-cell populations by PCR amplification. To evaluate the status of the transgene in the αβ T-cell population in the 14 and 22 lines, αβ T cells were enriched by the panning of thymocytes with the anti-δTCR antibody 403A10. Cells that were not adherent to the 403A10 antibody plates were classified as polyclonal αβ T cells in these studies. The isolated, purified γδ cells were expanded (by addition of murine recombinant IL-2 to the panning plates), grown for 7 days, and utilized as described below. Fluorescence-activated cell sorter analysis demonstrated >99% purity of the expanded γδ T-cell population (23).

As seen in Fig. 4a, δREC-ψJα recombinations are easily detected in the αβ T-cell populations of the thymus, spleen (data not shown), and even liver DNA. δREC-ψJα joints are a consistent finding in liver and probably represent contamination from circulating αβ T cells in blood.

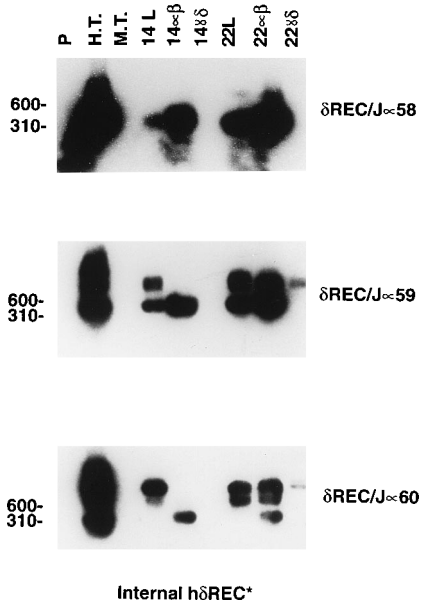
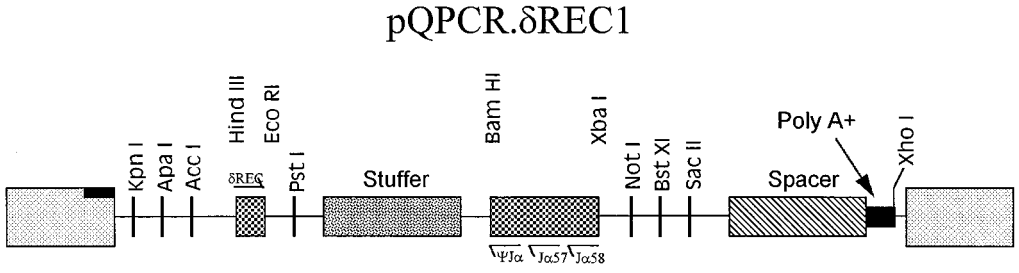


FIG. 5. Southern blot hybridized with radiolabeled human δREC probe of PCR products obtained with δREC, Jα58, Jα59, and Jα60 primers. P, primers only; H.T., human thymus DNA; M.T., nontransgenic murine thymus DNA; 14L, transgenic line 14 liver DNA; 14αβ, transgenic line 14 polyclonal thymus DNA with γδ T cells depleted; 14γδ, transgenic line 14 polyclonal thymic γδ T cells; 22L, transgenic line 22 liver DNA; 22αβ, transgenic line 22 polyclonal thymus DNA with γδ T cells depleted; 22γδ, transgenic line 22 polyclonal thymic γδ T cells. Molecular sizes are given to the left in base pairs.

However, δREC-ψJα recombinations are not detected or are barely detected in γδ T cells of lines 14 and 22. The same holds true for δREC-Jα_{57} (Fig. 4b) and three other human Jαs included in the construct (Fig. 5), even though DNA from all sources clearly can support PCR (Fig. 4c). The presence of δREC-3′ deleting element recombinations is not a consistent finding in the γδ population (23). Even when δREC-3′ deleting element recombinations are seen, the level is always much lower than that seen in liver DNA, and their presence likely represents contamination by αβ T cells from incomplete panning.

The two bands detected by an internal probe from the PCRs (Fig. 4b and Fig. 5) likely are a result of heteroduplex DNA formed when two strands anneal with as few as 3-bp differences (56). In this case, the mismatches are a result of different N segment additions within the δREC-Jα joints. The lower band represents the expected product, while the apparent higher-molecular-weight band contains the heteroduplexed DNA. The base mismatches cause loops to form after annealing, which causes the DNA to migrate more slowly in nondenaturing agarose gels. Alternatively, some of the larger bands may represent recombination into the adjacent Jα segment. For example, in Fig. 5, PCR with Jα_{59} may detect rearrangements into Jα_{59} or Jα_{60}, because they are separated by ≈250 bp.

Quantitative PCR indicates that rearrangements in the αβ T-cell population are 2,000-fold more frequent than in the γδ population. The submolar intensity of the δREC transgene rearrangements by Southern blot analysis (Fig. 3) might indicate that only a fraction of the T cells undergo rearrangement of the transgene. In order to better judge the frequency of rearrangement in the αβ T-cell population and more precisely compare αβ and γδ subsets, quantitative PCR for δREC-3′ δ deleting elements was developed. A competitor containing δREC and several Jα segments (Fig. 6) was constructed and utilized with the enzyme immunoassay procedure described previously (25). Figure 7a shows the analysis of δREC-ψJα, δREC-Jα_{57}, and δREC-Jα_{58} recombinations in the polyclonal T-cell population of the thymus. The IL-10 primers of construct pQPCR.MCC7 (25) are situated within a single exon of that gene and serve as a control for the number of alleles within each DNA preparation. Similarly, Fig. 7b illustrates δREC-Jα_{58} recombinations in the γδ T-cell population isolated from transgenic spleen. Table 3 summarizes the data and indicates that δREC recombinations are ≈2,000-fold more frequent in the purified CD8$^+$ T-cell population than in the purified γδ T-cell population. Splenic γδ T cells had no measurable δREC-ψJα or δREC-Jα_{57} rearrangements, with "not measurable" defined as <0.3 rearrangements per 100,000 IL-10 alleles in Table 3.



δREC Rearrangement	Endogenous Size	Competitor Size
ΨJα	539	285
Jα_{57}	421	309
Jα_{58}	371	319

FIG. 6. Schematic representation of quantitative PCR competitor pQPCR.δREC1. The sizes (base pairs) given for endogenous and competitor δREC rearrangements are approximate, because the degree of exonucleolytic and N segment addition activity will cause the absolute recombinant size to vary. Note that the spacer and poly(A)$^+$ tail are not utilized in a DNA competitor (25).

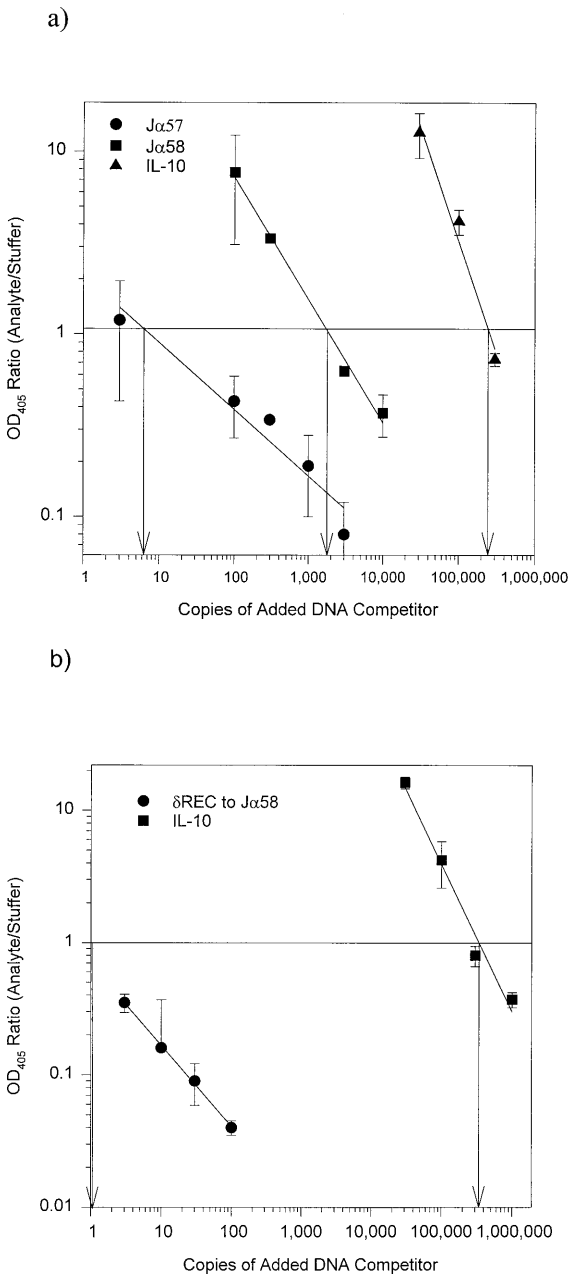


FIG. 7. Quantitative PCR of δREC-$Jα_{58}$, δREC-$Jα_{57}$, and δREC-ψJα recombinants in purified splenic $CD8^+$ αβ T cells (a) and purified splenic γδ T cells from transgenic animals of line 14 (b). Primers for IL-10 are part of competitor pQPCR.MCC7 (25), are contained within one exon of the IL-10 gene, and were used as DNA controls. OD_{405}, optical density at 405 nm.

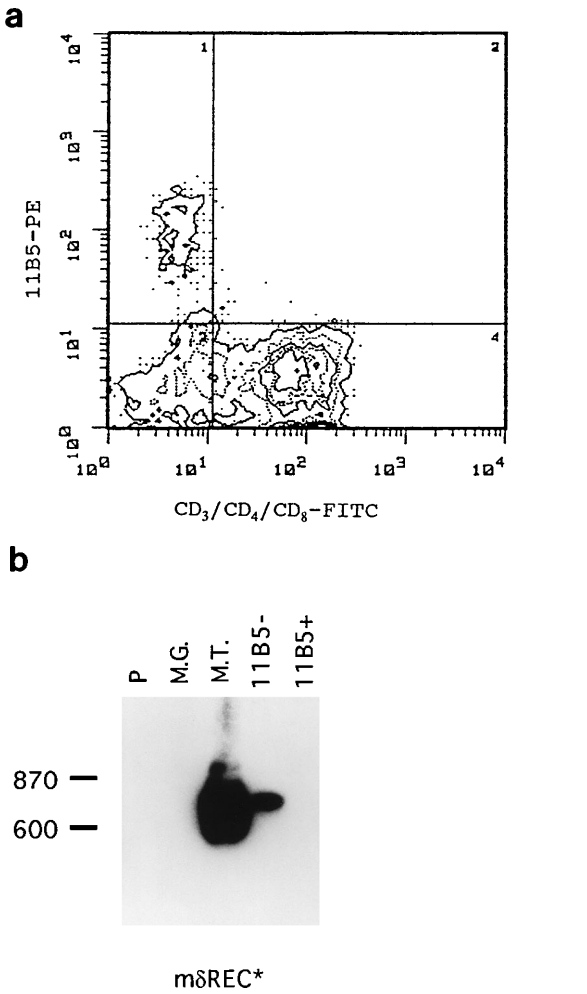


FIG. 8. Analysis of 11B5 sorted thymocytes. (a) Fluorescence-activated cell sorter scatter plot of CD3, CD4, and CD8 versus 11B5 on murine thymocytes. Cells were enriched by removal of $CD8^+$ cells prior to staining. CD3, CD4, and CD8 were FITC conjugated. 11B5 was biotinylated, with streptavidin-PE as the secondary reagent. Area R3 represents the triple-negative, $11B5^-$ sorted population, while area R1 represents the triple-negative $11B5^+$ sorted population. (b) Southern blot of PCR products from murine δREC-ψJα recombinations in 11B5 sorted populations of $CD3^-$ $CD4^-$ $CD8^-$ thymocytes probed with an internal murine δREC probe. P, primers only; M.G., murine placental DNA; M.T., murine thymus DNA. 11B5−, $CD3^-$ $CD4^-$ $CD8^-$ $11B5^-$ thymocytes; 11B5+, $CD3^-$ $CD4^-$ $CD8^-$ $11B5^+$ thymocytes. Molecular sizes are given to the left in base pairs.

δREC-ψJα recombinations are detected in developing thymocytes that have not yet had Vα rearranged to Jα. With the cell surface molecule 11B5, two populations of $CD3^-$ $CD4^-$ $CD8^-$ thymocytes have been sorted into $11B5^+$ and $11B5^-$ subsets (Fig. 8a). When thymus tissue from 3- to 6-week-old DBA/2 mice was used, approximately 500,000 $11B5^+$ positive and $11B5^-$ cells could be isolated. As shown in Fig. 8b, PCR analysis with δREC- and ψJα-specific primers has indicated that 11B5 delineates two unique populations with regard to that specific recombination. Utilization of the δ deleting elements is unique to the $11B5^-$ population.

The same DNA has been analyzed for the occurrence of all four TCR chain recombinations. As shown in Fig. 9, both $11B5^+$ and $11B5^-$ populations contain TCR β, γ, and δ chain rearrangements. These data are in agreement with published data about TCR status in early T-cell populations and the temporal expression of 11B5 (6, 16). However, Fig. 9 indicates that triple-negative thymocytes have not yet commenced TCR α chain rearrangement. Because the $11B5^-$ population of $CD3^-$ $CD4^-$ $CD8^-$ thymocytes contains δREC-ψJα recombinations, δ deletion occurs prior to Vα-Jα joining, at least on some alleles.

δREC-3′ δ deleting element transgenic rearrangements mirror the endogenous murine δ deleting element rearrangements. Because several recent reports have suggested that lineage-specific recombination can be governed by the presence of enhancers of either the TCR α chain or the TCR δ chain (7,

TABLE 3. Quantitation of δREC recombinants

Tissue	No. of recombinants[a]		
	δREC-ψJα	δREC-$Jα_{57}$	δREC-$Jα_{58}$
Thymus	3 ± 1	4 ± 2	100 ± 30
Spleen	0.5 ± 0.1	63 ± 12	21 ± 14
$CD8^+$ spleen	6 ± 2	6 ± 3	840 ± 160
γδ spleen	<0.3 ± ND[b]	<0.3 ± ND	0.3 ± 0.1

[a] Values have been normalized to 100,000 IL-10 alleles.
[b] ND, not determined.

33), we wanted to determine if the lineage-specific recombination of TG1 was under the control of the included murine Eα or human Eδ segments. To accomplish this, transgenic triple-negative thymocytes were sorted on the basis of 11B5 reactivity. As seen in Fig. 10, the pattern of transgenic δREC rearrangements parallels that seen for the endogenous murine δ deleting elements (Fig. 8 and 9). In particular, transgenic δREC-$Jα_{58}$ rearrangements are detected in the triple-negative, $11B5^-$ population, while endogenous $Vα_3$-$Jα_{19}$ rearrangements are not (Fig. 10). In agreement with the data presented above, endogenous $Vβ_8$ $Jβ_{1.3}$ recombinations are detected in both 11B5 sorted populations.

DISCUSSION

The δ chain locus is a gene within a gene, located in the much larger TCR α chain locus. This unusual organization of distinct rearranging loci necessitates a mechanism to control the utilization of the two receptors: αβ T cells do not incorporate internal δ chain segments in the α chain, and γδ T cells cannot allow subsequent α chain rearrangement with the resultant loss of the functional δ chain. We provide evidence that δ deletion is one means of implementing that choice.

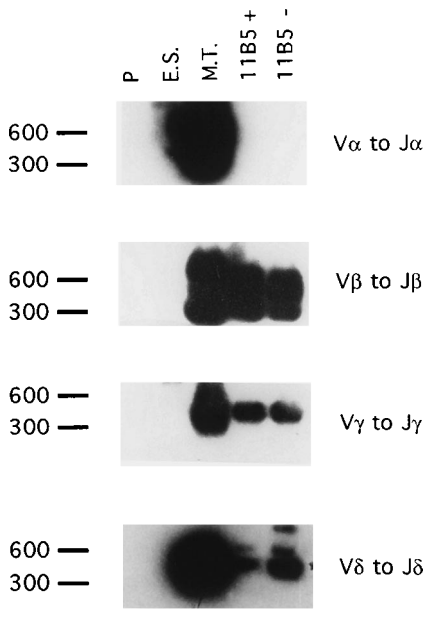


FIG. 9. Southern blot of PCR products from murine $Vα_3$-$Jα_{19}$, $Vβ_8$-$Jβ_{1.5}$, $Vγ_{1.2}$-$Jγ_2$, and $Vδ_4$-$Jδ_2$ rearrangements on 11B5 sorted populations of $CD3^-$ $CD4^-$ $CD8^-$ thymocytes probed with appropriate internal probes. P, primers only; E.S., embryonic stem cell DNA (negative control); M.T., murine thymus DNA; 11B5+, $CD3^-$ $CD4^-$ $CD8^-$ $11B5^+$ thymocytes; 11B5−, $CD3^-$ $CD4^-$ $CD8^-$ $11B5^-$ thymocytes. Molecular sizes are given to the left in base pairs.

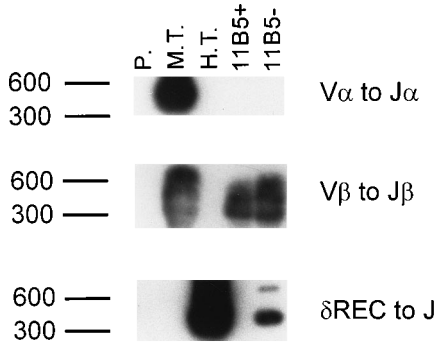


FIG. 10. Southern blot of PCR products from murine $Vα_3$-$Jα_{19}$, $Vβ_8$-$Jβ_{1.5}$, and human δREC-$Jα_{58}$ amplifications from 11B5 sorted, triple-negative thymocytes of transgenic line 14 probed with appropriate internal probes. P, primers only; M.T., murine thymus DNA; H.T., human thymus DNA; 11B5+, $CD3^-$ $CD4^-$ $CD8^-$ $11B5^+$ thymocytes; 11B5−, $CD3^-$ $CD4^-$ $CD8^-$ $11B5^-$ thymocytes. Molecular sizes are given to the left in base pairs.

The high degree of evolutionary conservation of both 5′ and 3′ δ deleting elements between mice and humans strongly suggests that these elements are important in T-cell development. δREC and ψJα are located in identical positions, have a remarkable degree of sequence identity, and appear to be utilized in the same manner (26). The only difference seems to be the frequency of specific δREC-ψJα recombinations in humans versus mice. We now demonstrate that murine δREC uses multiple Jαs as 3′ acceptor sites (Fig. 1). Because of the number of existing murine Jαs (n = 50 [29]) and other evidence suggesting that more than one δREC-like element also exists in the mouse (39, 48), the process appears to be the important phenomenon and not the specific elements.

The necessity for δ deletion prior to Vα-Jα joining has been difficult to demonstrate. Assessment of the configuration of deleting elements in individual cells as they mature is the ultimate best proof for this phenomenon. However, aside from showing that as an in vitro population, immature T cells progress to undergo δREC recombination prior to forming functional α TCR chains (13), this type of system is not available. However, the results from δ deleting element and TCR recombination on sorted populations of early T cells suggest that δREC-ψJα rearrangements occur prior to Vα-Jα joining, at least on some alleles. Data of this type support the model of δ deletion. The caveat is that relatively few Vα segments have been utilized for this analysis. If preferential utilization of Vα regions occurs, Vα-Jα rearrangements could be missed within the sorted populations. However, a total of two Vαs with two separate Jα segments have been used in PCR experiments, all combinations which are negative for Vα-Jα recombinations.

Additional support for the model of δ deletion was reported by Ohashi et al. (39). They found that when δREC3 is utilized as the 5′ deleting element, its subsequent deletion on the non-α-rearranged allele does not always occur, allowing the status of δ deleting elements to be determined. Germ line Cδ segments were not found in any αβ T-cell clone, supporting a model for δ deletion in αβ T cells. The status of δRECs on the α-rearranged allele could not be assessed, nor was the utilization of δREC elements in the γδ population examined.

We have designed a human transgenic reporter for the occurrence of δREC-ψJα recombinations in all mature T cells. The transgenic construct does not contain sufficient δ chain elements to form a complete receptor and is unlikely to alter physiology. Even though $Vδ_3$ could be rearranged to one of the Jα segments present, forming a hybrid Vδ-Jα rearrangement,

and then could be spliced to murine Cα, this rearrangement has not previously been reported. It is also doubtful that a human-murine α-δ hybrid molecule would significantly alter murine T-cell physiology and development. Surprisingly, this construct undergoes rearrangement in all founder lines that maintain orientation of the elements upon insertion (Table 2). These rearrangements involve the 5′ deleting element, δREC, and several 3′ deleting elements, ψJα and $Jα_{56–60}$. Interestingly, this finding recapitulates what happens on murine chromosome 14. Murine ψJα is not targeted, and the 3′ acceptor site uses any recombinatorial signal in the area.

While quantitative PCR suggests that not all αβ T cells undergo transgenic δREC rearrangement, there clearly is a lineage-specific occurrence of such events. At present, we cannot explain why $Jα_{58}$ and, to a lesser extent, $Jα_{57}$ are preferentially targeted in these transgenes. It seems curious that $Jα_{58}$ is selected when other h-s-n motifs are between it and ψJα (28). While δREC-ψJα can be seen by Southern blotting, and presumably the other faint bands in Fig. 3a represent recombinations to $Jα_{56–60}$, the δREC-$Jα_{58}$ recombination appears dominant in polyclonal thymus and purified splenic $CD8^+$ T-cell populations. Alternatively, it is possible that the majority of δREC rearrangements preferentially utilize $Jα_{56}$, $Jα_{59}$, or $Jα_{60}$ (as yet untested in quantitative PCR). This seems unlikely, given the similar degrees of reactivity on nonquantitative PCR in Fig. 4 and 5. However, to rule out this possibility, constructs containing these other Jα segments are being made.

Importantly, recombinations to $Jα_{57}$, $Jα_{58}$, or ψJα do not occur to any extent in the γδ T cells of the thymus. The occasional rearrangement detected by PCR likely represents contamination by αβ T cells in both the 14 and 22 lines (Fig. 4, 5, and 7b). The enumeration of these recombinants by quantitative PCR indicates that they represent a tiny fraction of the DNA, with only one δREC-$Jα_{58}$ recombinant detected in 0.5 μg of γδ genomic DNA (Fig. 7b). Even if they are true recombinations in γδ T cells, the disparity from that seen in the αβ population is clearly evident. The fact that the transgene undergoes rearrangement in αβ cells and does not, to any extent, in the γδ fraction implies specific mechanisms at areas other than the h-s-n recombinational signal which direct recombination.

One mechanism that appears to show lineage-specific recombination of transgenic substrates has been attributed to the Eα or Eδ elements. Capone et al. demonstrated αβ T-cell-specific rearrangements of V-D-J recombination when an Eα segment was included in the transgenic construct (7). The γδ T-cell population showed few if any transgenic substrate rearrangements. The recombinations when the Eα segment was present were also developmentally rearranged to the latter double-negative thymocyte populations. Similarly, Lauzurica and Krangel demonstrated that a transgenic V-D-J minilocus was rearranged in αβ T cells when the Eα was included within the minilocus and that the minilocus was rearranged in γδ T cells when the Eδ was included within the locus (33). Furthermore, the rearrangements under apparent Eδ control occurred prior to those under apparent Eα control within the developing thymus, matching very closely rearrangement patterns in the endogenous murine α-δ locus. These data suggest that when enhancer elements are included in a transgenic minilocus, they play an important role in governing lineage-specific developmental rearrangements.

Because construct TG1 contains the murine Eα segment, it is possible that the lineage-specific δREC rearrangements reported here are due to Eα effects instead of being under the control of δREC regulatory elements. To test this possibility, we sorted transgenic triple-negative thymocytes on the basis of 11B5 reactivity and demonstrated that the transgene is rearranged within the triple-negative, $11B5^-$ population, just as the endogenous δ deleting elements are (Fig. 9 and 10). Furthermore, neither transgenic triple-negative population has detectable Vα3-Jα19 rearrangements, suggesting that the Eα effects, if any, are minimal on the rearrangement of the transgenic locus.

Moreover, we have constructed a second transgene (TG2) that is identical to TG1, except that a 50-bp segment near human δREC was deleted (34). Removal of this 50-bp segment, which is recognized by putative regulatory proteins, results in transgenic δREC rearrangements in both αβ and γδ T-cell lineages (34). Since TG2 contains the same murine Eα, together with the evidence that the transgene is rearranged with the same temporal pattern to the endogenous murine TCR α-δ locus presented here, we conclude that lineage-specific rearrangements reported in TG1 are not due entirely to the presence of the murine Eα element. The differences between the findings of Capone et al. (7) and Lauzurica and Krangel (33) and those of our study might be due to the addition of another enhancer element in our construct, human Eδ, which ultimately may result in other elements playing a greater role in dictating lineage-specific rearrangement events. Alternatively, perhaps the effects of other nearby controlling regions, if present, take precedence over those of either enhancer. We propose that in our construct, HPSA1 is one such element (34).

The transgenic δ deleting element data presented are compatible with both models of T-cell development: precommitted lineage specificity (αβ or γδ) or progressive utilization of δ and then α rearrangements. The only implication is that δ deletion takes place prior to Vα-Jα joining. If inhibition of α recombinations occurs in γδ T cells, the putative controlling elements would still be present in failed δ chain rearrangements, and we favor a model that requires deletion of these undefined segments before α recombination can occur. Corroborating data from lineage commitment models from different sources are conflicting. Winoto and Baltimore's data about thymic circular DNA suggest precommitted lineages by demonstrating that deletional products from Vα-Jα joining do not contain internal Dδ-Jδ rearrangements (52). Additionally, Ohashi et al. (39) with αβ transgenic mice and Dent et al. (11) and Ishida et al. (27) with γδ transgenic mice have shown data that also suggest a precommitted lineage specificity, with the nontransgenic lineage developing normally. These latter data indicate that regardless of which TCR chains are expressed as a transgene, the developing cell has already decided to become one lineage of T cells or the other.

Alternatively, and in support of progressive rearrangement models of T-cell development, Thompson et al. demonstrated with hybridoma rescue of immature thymocytes that α chain and δ chain rearrangements may occur in individual clones on opposite alleles (50). Furthermore, Takeshita et al. (48), looking at thymic DNA circles from putative Vα-Jα joining events in contrast to Winoto and Baltimore (52), have found internal δ TCR recombinations. Finally, two recent reports (14, 35) demonstrate that many αβ T cells harbor TCR δ chain rearrangements, strongly suggesting that cells first attempt to rearrange the TCR δ chain and then progress to rearrange the TCR α chain genes. The finding of internal TCR δ chain genes also suggests that δ deletion has not occurred on both alleles. This could imply that δ deletion is not a requisite event in the formation of αβ T cells. It is possible, however, that δ deletion is an allele-specific requirement only and that the status of the opposite allele is independently controlled. Ultimately, the reasons for the discrepancies in these different models are not

clear but point to a potentially complex process which is not completely understood.

δ deletion as a necessary prelude to α chain recombination also does not discount the contribution of other elements contributing to α-δ separation. Silencer elements of both α and γ transcription may also regulate this process (27, 53). Our model does not identify the mechanism for inhibiting α recombination in γδ cells. This, in part, may involve the α silencer. Furthermore, the precise signals responsible for initiating TCR chain rearrangement and expression are not known. Early rearrangement and expression of the β and γ chains may affect the rearrangement and expression of corresponding α and δ chains (19, 55). However, our data clearly demonstrate that the machinery for utilizing the δ deleting elements is present in αβ T cells and absent in the γδ lineage. This is compatible with the model of δ deletion as part of the primary mechanism for implementing the signal to begin rearrangements within the TCR α chain locus and thus choosing the αβ T-cell lineage.

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