Enhancer-dependent and -independent Steps in the Rearrangement of a Human T Cell Receptor \(\delta\) Transgene

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Summary

The rearrangement and expression of T cell receptor (TCR) gene segments occurs in a highly ordered fashion during thymic ontogeny of T lymphocytes. To study the regulation of gene rearrangement within the TCR \(\alpha/\delta\) locus, we generated transgenic mice that carry a germline human TCR \(\gamma\) minilocus that includes \(V_{\delta}1, V_{\delta}2, D_{\delta}3, J_{\delta}1, J_{\delta}3,\) and \(C_{\delta}\) segments, and either contains or lacks the TCR \(\delta\) enhancer. We found that the enhancer-positive construct rearranges stepwise, first \(V\) to \(D\), and then \(V-D\) to \(J\). Construct \(V-D\) rearrangement mimics a unique property of the endogenous TCR \(\gamma\) locus. \(V-D-J\) rearrangement is T cell specific, but is equivalent in \(\alpha/\beta\) and \(\gamma/\delta\) T lymphocytes. Thus, either there is no commitment to the \(\alpha/\beta\) and \(\gamma/\delta\) T cell lineages before TCR \(\delta\) gene rearrangement, or if precommitment occurs, it does not operate directly on TCR \(\delta\) gene cis-acting regulatory elements to control TCR \(\delta\) gene rearrangement. Enhancer-negative mice display normal \(V\) to \(D\) rearrangement, but not \(V-D\) to \(J\) rearrangement. Thus, the \(V-D\) to \(J\) step is controlled by the enhancer, but the \(V\) to \(D\) step is controlled by separate elements. The enhancer apparently controls access to \(J_{\delta}1\) but not \(D_{\delta}3\), suggesting that a boundary between two independently regulated domains of the minilocus lies between these elements. Within the endogenous TCR \(\alpha/\delta\) locus, this boundary may represent the 5' end of a chromatin regulatory domain that is opened by the TCR \(\delta\) enhancer during T cell development. The position of this boundary may explain the unique propensity of the TCR \(\delta\) locus to undergo early \(V\) to \(D\) rearrangement. Our results indicate that the TCR \(\delta\) enhancer performs a crucial targeting function to regulate TCR \(\delta\) gene rearrangement during T cell development.

The ability of the immune system to recognize a diverse universe of antigens results in large part from the process of V-D-J recombination that assembles the genes encoding antigen receptors on T and B lymphocytes (1–3). Studies of lymphocyte development reveal that the assembly of antigen receptor genes is under stringent developmental control. The rearrangement of Ig genes occurs in a stepwise fashion during B cell maturation, with initial D to J joining followed by V to D-J joining at the H chain locus, and subsequently, V to J joining at either the \(\kappa\) or \(\lambda\) L chain locus (1). Similarly, T cell development is characterized by the ordered rearrangement and expression of TCR genes (4–6). The TCR \(\delta\) locus is the first to initiate rearrangement, at day 14 of murine fetal thymic development (7, 8). This is rapidly followed by rearrangement at the TCR \(\gamma\) and \(\beta\) loci, but TCR \(\alpha\) rearrangement does not begin until fetal day 17 (9–13). Furthermore, in mice and humans, the rearrangement of distinct \(V_{\delta}\) and \(V_{\delta}\) gene segments occurs in a stepwise fashion during fetal and early neonatal life (12, 14–17). This is the result of programmed rearrangement rather than cellular selection as a consequence of TCR expression, since TCR \(\delta\) gene mutant mice still undergo stepwise TCR \(\gamma\) and \(\delta\) gene rearrangement in the absence of surface TCR \(\gamma/\delta\) expression (18).

Progress has been made in understanding the mechanism of V-D-J recombination (2, 3). However, the manner in which complex patterns of gene rearrangement are orchestrated in developing lymphoid cells is only poorly understood. Immature lymphoid cells display recombinase activity that is dependent upon the expression of the RAG-1 and RAG-2 genes, and that is required for TCR and Ig gene rearrangement (19–22). Further, all TCR and Ig gene segments are flanked by conserved heptamer and nonamer recombination signal sequences that are essential substrates recognized by the recombinase machinery (23). However, recombinase activity and recombination signal sequences by themselves do not appear to provide the specificity implied by the temporal and lineage-restricted rearrangement of specific gene segments. Rather, such targeting appears to occur via modulation of substrate accessibility to the recombinase (1, 3). How this is accomplished is unclear; transcriptional activity, methylation status, and chromatin structure have all been associated with com-
Materials and Methods

Constructs. The transgene Vα1Vα2-D3-Jα1-Jα3-Ex-Cα was constructed by stepwise subcloning of previously described germline DNA fragments of the human TCR δ locus (35) into pBluescript KS+ (Stratagene, La Jolla, CA). In the process, all Xbal sites were destroyed except for two that flank Ex. Step 1: A 3.9-kb Xbal fragment containing D3 was treated with the Klenow fragment of Escherichia coli DNA polymerase I to generate blunt ends, and was cloned into similarly blunt-ended and phosphatase-treated Xbal-digested pBluescript. Step 2: the 10.5-kb BamHI-KpnI fragment containing J3, Ex, and Cα was ligated into BamHI- and KpnI-digested plasmid generated in step 1 to yield D3-J3-Ex-Cα. Step 3: The 1.8-kb Xbal-XbaI fragment containing J4 was blunt ended as above and cloned into BamHI-digested, blunt-ended, and phosphatase-treated plasmid from step 2 to yield D3-J3-J4-Ex-Cα. Step 4: A 3.1-kb XbaI-SalI fragment containing V2 cleaved in pBluescript was linearized within the V2 coding region by digestion with PvuI, treated with T4 polynucleotide to generate blunt ends, and religated to generate a 2-bp frame shift. Step 5: A 3.1-kb EcoRI fragment containing V1 cloned in pBluescript was linearized within the V1 coding region by digestion with BsmI, blunt ended by treatment with T4 polymerase, and phosphatase treated. A single 10-bp ClaI linker was then ligated into this site. Step 6: The mutated V2 fragment generated in step 4 was excised from the plasmid by digestion with Xbal and SalI, blunt ended by Klenow treatment, and cloned into Xbal-digested, blunt-ended, and phosphatase-treated V1 plasmid from step 5. Step 7: Insert containing V1 and V2 was excised from the step 7 plasmid by digestion with SalI and NotI, and was treated with Klenow to generate blunt ends. This fragment was ligated into NotI-digested, blunt-ended, and phosphatase-treated plasmid from step 3 to yield V1-V2-J1-D3-J3-Ex-Cα. Step 8: The enhancer-negative construct was generated from the step 7 plasmid by digestion with Xbal to liberate a 1.4-kb fragment containing Ex, followed by recircularization of the plasmid. Fragment orientations were confirmed at all steps either by digestion with appropriate restriction enzymes, or by nucleotide sequencing using appropriate primers. The V1 and V2 mutations were confirmed by nucleotide sequence analysis.

Production and Analysis of Transgenic Mice. Plasmids carrying the enhancer positive and negative constructs were purified by two rounds of CsCl density gradient centrifugation. Plasmids were digested with KpnI and KspI to liberate inserts of 22.5 (enhancer positive) and 21.1 kb (enhancer negative), which were purified by electrophoresis through 0.7% agarose (PurElute; Invitrogen, San Diego, CA) followed by electroelution. Eluted DNA was extracted four times with phenol/chloroform and once with chloroform, ethanol precipitated, and resuspended at 20 μg/mL. Fertilized C57BL/6 × SJL F2 eggs were microinjected with DNA and introduced into the oviducts of pseudopregnant C57BL/6 × SJL F1 females by the Duke University Comprehensive Cancer Center Shared Transgenic Mouse Facility. Progeny tail DNA was prepared by proteinase K digestion as described (43). EcoRI-digested DNA was analyzed on Southern blots using a radiolabeled Cα cDNA fragment to screen for integrated construct.

Polymerase Chain Reaction. Genomic DNA (0.6 μg) was amplified for 25 cycles in a 25-μl reaction containing 0.2 mM dATP, dCTP, dGTP, and 4 mM dUTP, 50 mM KCl, 3 mM MgCl2, 0.01% gelatin, 100 mM Tris-HCl (pH 8.3), 0.25 U Taq polymerase, 0.2 U Uracil N-glycosylase, and 20 pmol of each primer oligonucleotide (44). Each cycle consisted of a 1-min annealing step at 94°C, 1-min annealing at 56°C, and 2-min extension at 72°C. One fifth of each reaction was analyzed by agarose gel electrophoresis through 0.7% agarose (PurElute; Invitrogen, San Diego, CA) followed by electroelution. Electrophoresed DNA was extracted four times with phenol/chloroform and once with chloroform, ethanol precipitated, and resuspended at 20 μg/mL. Fertilized C57BL/6 × SJL F2 eggs were microinjected with DNA and introduced into the oviducts of pseudopregnant C57BL/6 × SJL F1 females by the Duke University Comprehensive Cancer Center Shared Transgenic Mouse Facility. Progeny tail DNA was prepared by proteinase K digestion as described (43). EcoRI-digested DNA was analyzed on Southern blots using a radiolabeled Cα cDNA fragment to screen for integrated construct.

Enhancer Control of TCR δ Gene Rearrangement
Nucleotide sequences were obtained after cloning of amplified products into pBluescript KS+ as described previously (16). Blot Hybridization of Genomic DNA and PCR Products. Genomic DNA was prepared from tissue and cell suspensions by proteinase K digestion according to established procedures (45). Gel electrophoresis, blotting, hybridization with \(^{32}P\)-labeled probes, and washing were as previously described (46). The probes used were: 5'J\(_{11}\), a 0.4-kb XbaI-PstI fragment mapping 5' of J\(_{11}\) that was isolated from the genomic 1.8-kb J\(_{11}\) XbaI fragment; J\(_{11}\), a 1.4-kb PstI-XbaI fragment isolated from the same fragment; and 5' D\(_{3}\), a 0.5-kb HindIII-PstI fragment isolated from the genomic 3.9-kb XbaI fragment carrying D\(_{3}\). The C\(_{\alpha}\), V\(_{\alpha}1\), and V\(_{\alpha}2\) probes have been described previously (32, 46). All probes were radiolabeled by the method of random hexamer priming (47). Quantitative analysis of blots was accomplished using a betascope (Betagen, Waltham, MA).

Antibodies and Flow Cytometry. H57-597 (biotin-conjugated hamster anti-mouse TCR \(\alpha/\beta\) IgG), GL3 (R-PE-conjugated hamster anti-mouse TCR \(\gamma/\delta\) IgG), 30-H12 (rat anti-mouse Thy-1.2 IgG2b), 53-6.7 (rat anti-mouse CD8 IgG2a), GK1.5 (rat anti-mouse CD4 IgG), Fe Block (rat anti-mouse Fc\(\gamma\)II receptor [CD32]), biotin-conjugated hamster IgG isotype control, and streptavidin-PE were obtained from Pharmingen (San Diego, CA). Affinity-purified, R-PE conjugated goat anti-hamster IgG F(ab')\(_2\) fragment was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). FITC-labeled goat anti-mouse IgG and FITC-labeled goat anti-rat IgG were purchased from Dako Corp. (Carpinteria, CA).

For flow cytometry, 1–2 \(\times\) 10\(^6\) cells were washed three times in 2% BSA and 0.1% sodium azide in PBS and were incubated in 50 \(\mu\)l of the same buffer containing 20 \(\mu\)g/ml of appropriate Ab for 30 min at 4\(^\circ\)C. Cells were washed three times and were then resuspended in 50 \(\mu\)l of the same buffer containing the appropriate secondary step reagent for a 30-min incubation at 4\(^\circ\)C in the dark. Cells were again washed three times, resuspended in the same buffer with 1% paraformaldehyde, and were analyzed using a FACScan® (Becton Dickinson & Co., Mountain View, CA).

Cell Isolation. Enriched splenic T cell preparations were generated by passage of spleen cell suspensions over a nylon wool column as described (48). T cells were further purified by staining with anti-Thy-1.2 and FITC-conjugated goat anti-rat IgG followed by cell sorting using a FACStar® (Becton Dickinson & Co.). Enriched splenic B cells were generated by two rounds of T cell depletion using anti-Thy-1.2 plus rabbit complement (48). B cells were further purified by staining with FITC-labeled goat anti-mouse F(ab')\(_2\) followed by cell sorting. Thymic \(\alpha/\beta\) and \(\gamma/\delta\) T cells were purified by two-color staining of thymocytes (10\(^7\) cells/ml) using biotin-conjugated H57-597 and PE-conjugated GL3 (5 \(\mu\)g/ml each), followed by FITC-streptavidin and cell sorting. In some experiments, thymocytes were enriched for CD4–8– cells by two rounds of depletion with 53–6.7 and GK1.5. The purity of sorted populations was assessed by immediate reanalysis of the sorted sample using a FACScan®.

Human Cell Samples. TCR \(\delta\) gene rearrangements in the T cell leukemia Molt-13 (46), fetal thymocyte clone Solo-15 (16), and a 12-wk fetal thymocyte T cell culture (16) were previously characterized. A polyclonal V\(_{\alpha}2^+\) culture of \(\gamma/\delta\) T cells from a 16-wk fetal liver was kindly provided by Dr. Hergen Spits (DNAX Research Institute, Palo Alto, CA).

Results

Strategy. We constructed a rearrangement substrate from a series of genomic clones carrying germline elements of the human TCR \(\delta\) locus (Fig. 1). These included a 3.1-kb fragment carrying V\(_{\alpha}1\), a 3.1-kb fragment carrying V\(_{\alpha}2\), a 3.9-kb fragment carrying D\(_{3}\), a 1.8-kb fragment carrying J\(_{\alpha}1\), and a 10.5-kb fragment carrying J\(_{\alpha}3\) and C\(_{\alpha}\) (35, 46). The latter fragment also contains the previously characterized TCR \(\delta\) transcriptional enhancer within the J\(_{\alpha}3\)-C\(_{\alpha}\) intron (49). D\(_{3}\) was included in the construct because it is the single human D\(_{3}\) segment that is almost universally used in rearranged TCR \(\delta\) genes (16, 17, 34). The V\(_{\alpha}1\) and V\(_{\alpha}2\) gene segments were both included within the construct because they appear to be differentially activated during human fetal thymic development (16, 17). V\(_{\alpha}2\) rearrangements are abundant in very early fetal thymocytes, whereas V\(_{\alpha}1\) rearrangements predominate in late fetal and neonatal thymocytes. Further, V\(_{\alpha}2\) usage is restricted to \(\gamma/\delta\) cells, whereas V\(_{\alpha}1\) is used in both \(\gamma/\delta\) and \(\alpha/\beta\) cells (50).

We wanted the construct to serve as an innocuous reporter that would not influence the rearrangement of endogenous TCR genes via the process of allelic exclusion. Therefore, mutations were introduced into both V\(_{\alpha}1\) and V\(_{\alpha}2\) to destroy their open reading frames and prevent a rearranged transgene from encoding a functional TCR protein. We also wanted to be able to easily remove the TCR \(\delta\) enhancer from the

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**Figure 1.** Schematic representation of the human TCR \(\delta\) minilocus. The transgene (top) was constructed from segments of the human TCR \(\delta\) locus (bottom). (Rectangle) The exons of V, D, J, and C gene segments. (Diamond) The TCR \(\delta\) transcriptional enhancer. Mutations were introduced into the V gene segments to prevent TCR protein expression from rearranged transgenes.
final construct, and have the flexibility to clone other regulatory elements in its place. Therefore, all XbaI sites within the construct were destroyed during the process of subcloning (see Materials and Methods), except for two sites that lie between Jα3 and Cδ and flank the enhancer. Finally, we used oligonucleotide primers specific for Vα1, Vα2, Jα1, and Jα3 to analyze transgene V-D-J rearrangement by the PCR. Primers were positioned such that any V-D-J rearrangement would yield a PCR product of ~0.3 kb using the appropriate pair of V and J primers. Specific PCR products would not be generated from unarranged templates because of the much larger distances between primers.

**V-D and V-D-J Rearrangement of an Enhancer-positive TCR δ Minilocus.** The linearized 22.4-kb enhancer-positive construct was microinjected into fertilized C57BL/6 × SJL F2 eggs. Southern blot analysis identified three founders that carried a single copy of the minilocus (data not shown). One of these, founder F62, carried an intact minilocus as judged by EcoRI digestion and analysis with Vα1, Vα2, and Cδ probes. Another, founder F57, carried a minilocus that was disrupted at the 3' end. Of two EcoRI fragments detected by a Cδ probe, the 5' fragment appeared normal, but the 3' fragment was altered. Since the results obtained with mice derived from these founders were qualitatively similar (see below), the structural alteration in the 3' portion of Cδ does not significantly influence construct rearrangement. A third founder, F63, carried a minilocus with a 5' truncation that deleted the Vα1 gene segment, but retained the Vα2 gene segment. Founders were mated with C57BL/6 × SJL F1 mice to establish three transgenic lines (line A, derived from F57; line B, derived from F62; and line C, derived from F63). We noted that the transgene was inherited by all males in line B, suggesting that the transgene had integrated into the Y chromosome. Lines A and B were used for most of the experiments reported in this study.

To assess transgene rearrangement, genomic DNA was prepared from various tissues of two mice in each of transgenic lines A and B. PCR was performed for 25 cycles using appropriate pairs of V and J primers. The products were electrophoresed through agarose gels, transferred to nylon membranes, and hybridized with radiolabeled cDNA probes specific for the human Vα1 and Vα2 gene segments for detection. Parallel samples were analyzed by PCR, with a pair of Cδ primers, and were detected with a radiolabeled Cδ probe. These primers amplify a 0.3-kb fragment that is independent of construct rearrangement and therefore serves as a control for the efficiency of PCR in different DNA samples. The combinations of PCR primers and radiolabeled probes used detect rearrangements of the human TCR δ transgene, but do not detect rearrangements of the endogenous murine TCR δ gene (data not shown).

As can be seen in Fig. 2, each pair of V and J primers amplified products that were enriched in the thymus of neonatal mice (A-26 and B-29) and in the thymus and spleen of older mice (A-48 and B-31). High levels of PCR products in neonatal thymus and low levels in neonatal spleen could be consistent with T cell–specific rearrangement, since there are only small numbers of T lymphocytes in the spleen at this age (data not shown). The low level of PCR products detected in other tissues is most likely attributable to blood contamination. PCR using Cδ primers did not reveal significant differences in PCR efficiency from the various DNA sources within each panel.

The most abundant PCR product detected in thymus DNA with each primer pair was approximately 0.3 kb, consistent with the expected size of a V-D-J rearrangement. However, using the primer combinations Vα2-Jα1 and Vα1-Jα1, we also detected a product of 1.2 kb (Figs. 2 and 3 A). A product of this size is predicted to result from a partial rearrangement of Vα1 or Vα2 to Dα3. Because Dα3 and Jα1 are separated by only 0.9 kb in the germline configuration, the combination of a Vα1 or Vα2 primer with a Jα1 primer could amplify a V-D rearranged fragment of 1.2 kb that contains the 0.9-kb genomic segment between Dα3 and Jα1. Whereas the Vα1 probe hybridized with both the 1.2- and 0.3-kb fragments, the 5' Jα1 probe selectively hybridized with the 1.2-kb fragment, thereby identifying it as a Vα1-Dα3 rearrangement (Fig. 3 A). The partial Vα1-Dα3 and Vα2-Dα3 rearrangements are likely to be intermediates in the process of transgene V-D-J rearrangement.

Transgene V-D-J and V-D rearrangement was a consistent property of the minilocus in independent lines of transgenic mice. Using Vα2 and Jα1 primers, we detected Vα2-Dα3-Jα1 and Vα2-Dα3 rearrangements not only in line A and line B thymus samples, but in line C thymus samples as well (Fig. 3 C). Furthermore, the same primer pair was used to identify abundant Vα2-Dα3-Jα1 and Vα2-Dα3 rearrangements in T cell samples derived from human fetal liver and human fetal thymus (Fig. 3 C). Thus, as revealed by this PCR analysis, transgene rearrangement parallels the rearrangement of the endogenous human TCR δ locus.

A second potential intermediate in transgene rearrangement is D-J. We could not detect a 0.3-kb fragment representing Dα3-Jα1 rearrangement by PCR using 5' Dα3 and Jα1 primers, even though these primers did amplify a 1.2-kb fragment originating from unarranged chromosomes (data not shown). Furthermore, we did not detect the predicted Dα3-Jα1 fragment using a Jα1 probe to analyze Southern blots (see below). We therefore conclude that the transgene rearranges stepwise, and that the predominant pathway involves early rearrangement of V to D, and subsequent rearrangement of V-D to J. The identification of abundant transgene V-D rearrangement intermediates is quite striking, because rearrangements at the Ig H chain locus and at the TCR δ locus are well documented to proceed exclusively via D-J intermediates (1). However, studies of murine fetal thymocyte hybridomas (8) and human leukemias (51, 52) have shown that early V-D rearrangement is a unique property of the endogenous TCR δ locus (Fig. 3 C), and this property is clearly mimicked by the transgene. The rearrangement of the transgene differs slightly from the rearrangement of the en-
dogenous TCR δ locus because although we did not detect D-J rearrangement of the transgene, such rearrangements were detected at low levels in populations of human fetal liver and fetal thymus T cells (data not shown) and are well documented in populations of murine thymocytes (8, 53).

Quantification of Transgene V-D-J and V-D Rearrangement. To quantify transgene V-D-J rearrangement, we used as standards two human γ/δ T cell clones, each of which has a well-characterized, single-copy TCR-δ gene rearrangement of interest. Serially diluted A-48 thymus and human cell line DNA samples were subjected to PCR, electrophoresis, blotting and probing as described above, and V-D-J signals were then quantified (Fig. 3). Signal strengths were linearly related to the amount of input DNA (except at the highest concentrations of human cell line DNA), indicating that the data could be interpreted in a quantitative manner.

By this analysis, about 30% of the transgenes displayed Vδ1-Dδ3-Jδ1 rearrangements, and about 3% of the transgenes...
displayed Vδ2-Dδ3-Jδ3 rearrangements. This difference is not reflected in Fig. 2 because autoradiographic exposures of panels developed with the Vδ2 probe were adjusted to those developed with the Vδ1 probe, so that rearrangement specificity in lymphoid and nonlymphoid tissues could be compared within each panel. A more accurate reflection of the relative abundance of different rearrangements in line A thymus DNA is presented in Fig. 9 in which the specific activities of the probes and the autoradiographic exposure times were matched. From these experiments we estimate that Vδ1-Dδ3-Jδ3 and Vδ2-Dδ3-Jδ3 rearrangements are nearly as abundant as Vδ1-Dδ3-Jδ1 and Vδ2-Dδ3-Jδ3 rearrangements, respectively. Thus, assuming that the transgene is present as a single-copy integration, transgene V-D-J rearrangement is detected in ~60–70% of developing thymocytes in line A. The level of rearrangement in line B is ~20% of that in line A (see Fig. 3 C; this difference is not reflected in Fig. 2 because autoradiographic exposures were adjusted).

We could not reliably quantify transgene V-D rearrangements by PCR because we did not have single-copy Vδ1-Dδ3 and Vδ2-Dδ3 rearrangement controls for comparison. Therefore, we attempted to quantify the rearrangements by analyzing Southern blots of PstI- plus EcoRI-digested A-26 thymus DNA using Vδ1 and Jα1 probes (Fig. 5). We detected an abundant rearranged fragment of 1.7 kb with both the Vδ1 and Jα1 probes and an equally abundant fragment of 3.2 kb with the Vδ1 probe, as predicted for Vδ1-Dδ3-Jδ1 and Vδ1-Dδ3-Jδ3 rearrangements, respectively. Further, we identified another rearranged fragment of 0.9 kb with the Vδ1 probe, as predicted for Vδ1-Dδ3. By comparing the intensities of

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**Figure 4.** Quantification of TCR δ minilocus V-D-J rearrangement. Serially diluted samples of A-48 thymus DNA, human γ/δ T cell Molt-13 DNA, and human γ/δ T cell Solo 15 DNA were subjected to PCR using the indicated primer pairs and analyzed on Southern blots using Vδ1 and Vδ2 probes. Amplified 0.3-kb products were quantified using a betascope.

**Figure 5.** TCR δ minilocus rearrangement analyzed directly on Southern blots. (A) PstI-plus EcoRI-digested A-26 tail DNA (germline [G]) and thymus DNA (rearranged [R]) was analyzed on Southern blots using radiolabeled Jα1 and Vδ1 probes. Similarly digested H-46 tail DNA (G) and thymus DNA (R) was analyzed on a Southern blot using a radiolabeled Vδ1 probe. The expected germline (GL) and rearranged fragments are indicated along with their predicted sizes. Rearrangements involving Vδ2 occur at much lower frequency than those involving Vδ1, and are therefore not considered. The expected size of a Vδ2-Dδ3-Jδ1 rearrangement that might be detected with the Jα1 probe is 2.9 kb. (B) Vδ1, Dδ3, Jδ1 and Jδ3 segments, PstI and EcoRI sites, and Vδ1 and Jα1 probes are mapped in the enhancer-positive minilocus.
thymus DNA are compared with germline sequences using V\textsubscript{1} and J\textsubscript{1} or V\textsubscript{2} and J\textsubscript{1} primers from fetal day 17 and A-26 neonatal thymocytes. Junctional sequences of 0.3-kb PCR products amplified (Left) Numbers indicate V\textsubscript{1} segment used. Palindromic "P" nucleotides and template independent "N" nucleotides are identified.

\textbf{GERMLINE:}
\begin{center}
\begin{tabular}{ll}
GERMLINE: & NEONATAL A-26: \\
1 TTGG & 2 CTGTGACA \\
1 TTGGGAA & 2 CTGTGACCC \\
1 TTGGG & 2 CTGTGAC \\
1 TTGGGG & 2 CTGTGACACE \\
1 TTGGGGAA & 2 CTGTGACAC \\
1 TTGGGG & 2 CTGTGACACCE \\
1 TTGGGGAA & 2 CTGTGACAC
\end{tabular}
\end{center}

\textbf{FETAL DAY 17:}
\begin{center}
\begin{tabular}{ll}
FETAL DAY 17: & NEONATAL A-26: \\
1 TTGG & 2 CTGTGACA \\
1 TTGGGAA & 2 CTGTGACCC \\
1 TTGGG & 2 CTGTGAC \\
1 TTGGGG & 2 CTGTGACACE \\
1 TTGGGGAA & 2 CTGTGACAC \\
1 TTGGGG & 2 CTGTGACACCE \\
1 TTGGGGAA & 2 CTGTGACAC
\end{tabular}
\end{center}

\begin{itemize}
\item The signals for the rearranged fragments detected with the V\textsubscript{1} probe, we estimate that \~15% of the V\textsubscript{1} rearrangements in A-26 thymus are partial V\textsubscript{1}-D\textsubscript{3} rearrangements. Consistent with the PCR data, partial D\textsubscript{3}-J\textsubscript{1} rearrangements of 4.1 kb were not detected.

\item Fine Structure Analysis of V-D-J Junctions. The recombination signal sequences 3' of V\textsubscript{5} and D\textsubscript{5} gene segments display 23-bp spacers, whereas those 5' of D\textsubscript{5} and J\textsubscript{1} gene segments display 12-bp spacers (33-35). Thus, both V-D-J and direct V-J rearrangement would be allowed according to the 12/23 rule. Because of the small size of the D element, PCR products derived from these two classes of rearrangements would be indistinguishable by agarose gel electrophoresis. To determine whether the 0.3-kb fragments indeed represented V-D-J rearrangements typical of rearranged endogenous TCR \(\delta\) genes, the PCR fragments were cloned and then subjected to nucleotide sequence analysis. The V-D-J junction sequences of eight clones derived from fetal thymus and nine clones derived from neonatal thymus were determined (Fig. 6). All of the junctions show evidence of D segment usage. Further, the junctions display all the hallmarks of typical TCR \(\delta\) V-D-J rearrangements, including the evidence of P nucleotides, exonucleolytic digestion of coding ends, and incorporation of template independent N nucleotides (14, 16, 34, 54). As is the case for endogenous TCR \(\delta\) V-D-J junctions, and in accord with the known pattern of terminal transerase expression (55, 56), N nucleotides were absent from fetal thymocyte junctions (14, 16, 17, 54). Rather, all nucleotides in these junctions could be assigned as encoded by germline elements or as palindromic P nucleotides that are thought to result from the resolution of hairpin structures at the coding ends (54, 57). In contrast, N nucleotide incorporation was extensive in the junctions of neonatal thymocytes.

\item Lineage Specificity of TCR \(\delta\) Minilocus Rearrangement. Since transgene rearrangement is detected at high levels only in thymus and spleen (Fig. 2), it must, at a minimum, be lymphoid specific. To establish whether rearrangement occurs selectively in T lymphocytes, T and B cell populations were purified from line A and B spleens by a combination of cytotoxic elimination and cell sorting. PCR analysis using V\textsubscript{1} and J\textsubscript{1} primers revealed transgene V-D-J rearrangement to be abundant in splenic T cells, but undetectable in splenic B cells (Fig. 7 A). Transgene V-D rearrangement was highly enriched in splenic T cells, but could nevertheless be detected at low levels in splenic B cells. This was true both in the B cell preparation from mouse A-45, where the 1.2-kb V-D product was readily detected, and in the B cell preparation from mouse B-129, where this fragment was detected on longer exposures of the autoradiogram. This is clearly not due to residual T cell contamination, because the specificity of V-D-J rearrangement appeared absolute. Similar analysis of V\textsubscript{1}-J\textsubscript{3}, V\textsubscript{2}-J\textsubscript{1}, and V\textsubscript{2}-J\textsubscript{3} rearrangements indicated that transgene V-D-J rearrangement was T cell specific in all instances (Fig. 7 B).

We next sought to determine whether transgene rearrangement occurred equivalently in \(\alpha/\beta\) and \(\gamma/\delta\) T cells, or occurred preferentially in one of these cell populations. A combination of cytotoxic elimination and cell sorting was used

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\item \textbf{Lineage Specificity of TCR \(\delta\) Minilocus Rearrangement.} Since transgene rearrangement is detected at high levels only in thymus and spleen (Fig. 2), it must, at a minimum, be lymphoid specific. To establish whether rearrangement occurs selectively in T lymphocytes, T and B cell populations were purified from line A and B spleens by a combination of cytotoxic elimination and cell sorting. PCR analysis using V\textsubscript{1} and J\textsubscript{1} primers revealed transgene V-D-J rearrangement to be abundant in splenic T cells, but undetectable in splenic B cells (Fig. 7 A). Transgene V-D rearrangement was highly enriched in splenic T cells, but could nevertheless be detected at low levels in splenic B cells. This was true both in the B cell preparation from mouse A-45, where the 1.2-kb V-D product was readily detected, and in the B cell preparation from mouse B-129, where this fragment was detected on longer exposures of the autoradiogram. This is clearly not due to residual T cell contamination, because the specificity of V-D-J rearrangement appeared absolute. Similar analysis of V\textsubscript{1}-J\textsubscript{3}, V\textsubscript{2}-J\textsubscript{1}, and V\textsubscript{2}-J\textsubscript{3} rearrangements indicated that transgene V-D-J rearrangement was T cell specific in all instances (Fig. 7 B).

\item We next sought to determine whether transgene rearrangement occurred equivalently in \(\alpha/\beta\) and \(\gamma/\delta\) T cells, or occurred preferentially in one of these cell populations. A combination of cytotoxic elimination and cell sorting was used
to isolate purified populations of α/β and γ/δ T cells from neonatal thymus. PCR analysis using V\(_\delta 1\) and J\(_\delta 1\) primers revealed essentially equivalent levels of V-D and V-D-J rearrangements in γ/δ and α/β T lymphocytes (Fig. 8). This experiment was repeated with additional analysis of V\(_\delta 1\)-J\(_\delta 3\), V\(_\delta 2\)-J\(_\delta 1\), and V\(_\delta 2\)-J\(_\delta 3\) rearrangements, without any evidence for an enrichment of transgene rearrangement in the γ/δ T cell population (data not shown). Cross-contamination of cell populations was <1% when sorted populations were reanalyzed by flow cytometry. Minilocus rearrangement in α/β cells is consistent with our observation that a large fraction of neonatal thymocytes rearrange the transgene (Fig. 4), since <1% of neonatal thymocytes express a TCR γ/δ. These results indicate that rearrangement of the TCR δ transgene is not under the control of γ/δ lineage-specific signals in developing thymocytes. Rather, it has the potential to rearrange in precursors of both γ/δ and α/β cells.

**Rearrangement of an Enhancer-negative TCR δ Minilocus.** To determine whether the TCR δ enhancer plays an important role in regulating the rearrangement process, plasmid containing the enhancer-positive minilocus construct was modified by XbaI digestion followed by recircularization to delete the 1.4-kb fragment that includes the enhancer. The linearized 21-kb enhancer-negative minilocus construct was then used to generate transgenic mice as above. Four independent founders were identified that carried intact versions of the enhancer-negative minilocus. Three of these, F18, F19, and F22, carried single-copy integrations, whereas one, F25, carried multiple copies of the minilocus. Progeny derived from F25 segregated two different integration sites, one with a single copy, and one with two to three copies of the minilocus. As a result, a total of five different enhancer-negative transgenic lines were derived from the four founders. These were designated lines E (from F22), F (from F18), G (from F19), H (from F25 multicopy integration), and I (from F25 single-copy integration).

The analysis of minilocus rearrangement in thymus DNA from neonatal mice in each of these transgenic lines is presented in Fig. 9. Thymus DNA from enhancer-positive line A served as a control. In this experiment, V\(_\delta 1\) and V\(_\delta 2\) probes were matched for specific activity, and autoradiographic exposure times were identical, allowing for a meaningful comparison of the relative frequencies of the different rearrangements. Three different rearrangement phenotypes were discerned among the enhancer-negative mice. The most common phenotype was shared by lines E, F, and H. In these mice, minilocus V-D rearrangement appeared to occur normally. Strikingly, however, V-D-J rearrangement was not detected, indicating that the V-D to J step was selectively impaired. This PCR data was confirmed by an analysis of PstI plus EcoRI digests of line H DNA on Southern blots using a V\(_\delta 1\) probe (Fig. 5). A V\(_\delta 1\)-D\(_\delta 3\) rearranged fragment of 0.9 kb was readily detected, but V\(_\delta 3\)-J\(_\delta 1\) and V\(_\delta 1\)-D\(_\delta 3\)-J\(_\delta 1\) rearranged fragments of 1.7 and 3.2 kb, respectively, were not detected, even on longer exposures of the autoradiograph. Confirmatory Southern blot data were also obtained for transgenic line F (data not shown).

Although the level of total V rearrangement (i.e., V-D + V-D-J) varied among these lines of mice, the observed inhibition of the V-D to J step is independent of this variation. We estimate from the Southern blot in Fig. 5 that 80–90% of V\(_\delta 1\) gene segments had rearranged (V-D + V-D-J) in line A thymus. By comparison, in line H thymus fully 50% of V\(_\delta 1\) gene segments had undergone V\(_\delta 1\)-D\(_\delta 3\) rearrangement, without detectably proceeding to V-D-J rearrangement. Further, we estimate from PCR that the V\(_\delta 1\) and V\(_\delta 2\) rearrangement signals in line F were 17 and 22% of those in line A, respectively. This level of rearrangement is comparable with that in the enhancer-positive line B (20% of line A as quantified in this experiment [data not shown] and in Fig. 3 C). However, V-D-J rearrangement is readily detected in line B but not in line F.

Distinct phenotypes were displayed by lines G and I. In line G, no rearrangement was detected. However, in line I,
significant levels of V-D-J rearrangement were detected, albeit at frequencies that were reduced compared with those in the enhancer-positive line A. Because V-D and V-D-J rearrangement still occurred in the enhancerless construct in line I, we analyzed T and B lymphocyte populations purified from line I splenocytes to ask whether the T cell specificity of rearrangement was retained or lost. We found that V-D-J rearrangement in line I was still T cell specific, and that V-D rearrangement was still highly enriched in T cells (data not shown). The simplest interpretation of this observation is that there are regulatory elements within the construct other than the TCR δ enhancer that can function to restrict rearrangement to T cells.

The analysis of additional animals in each of enhancer-negative lines E-I indicated that the qualitative phenotypic differences between the mice analyzed in the above experiment are reproducible properties of the respective lineages. We conclude from these experiments that transgene V to D rearrangement is essentially normal in the absence of the TCR δ enhancer, whereas V-D to J rearrangement is severely impaired. We think that the quantitative differences in the level of transgene rearrangement in the enhancer-positive and -negative lines, as well as the phenotypic variability among the enhancer-negative lines, most likely results from the dominant effects of the different transgene integration sites. Such position effects on transgene expression are common unless locus control regions are included within transgene constructs (58–61). We suspect that the integration site in line G is particularly inert and functions to repress transgene rearrangement, whereas the integration site in line I is particularly active and partially reverses the enhancer-negative phenotype. Nevertheless, three of three enhancer-positive lines undergo V-D-J rearrangement, whereas three of five enhancer-negative lines undergo V-D rearrangement but do not proceed to V-D-J rearrangement. These results argue persuasively that the TCR δ enhancer performs a crucial targeting function that regulates a specific step in TCR δ gene rearrangement during T cell development.

Discussion

To begin to dissect the regulation of TCR gene rearrangement during T cell development, we generated transgenic mice with integrated copies of a human TCR δ minilocus. The minilocus contained selected segments of the endogenous TCR δ locus, including the Va1, Va2, Dα3, Jα1, Jα3, and Ca genes. Transgene V-D-J rearrangement was found to occur exclusively in T lymphocytes. In this and other respects, transgene rearrangement conserved significant features of the rearrangement of the endogenous TCR δ locus. Thus, although large portions of the endogenous locus are excluded from the minilocus construct, important cis-acting elements that control the rearrangement process are apparently conserved.

Because we detected high levels of transgene V-D and V-D-J rearrangements, but could not readily identify transgene D-J rearrangements, our data indicate that the minilocus rearranges in stepwise fashion, first V to D, and then V-D to J. The propensity for minilocus V-D rearrangement mimics a unique property of the endogenous TCR δ locus that is not shared with the TCR β and IgH loci. TCR β and IgH rearrangements are highly ordered, with D to J joining occurring first (1, 3). However, multiple lines of evidence argue that V-D and D-D joining events predominate among the early rearrangements at the endogenous human and murine TCR δ loci. V-D and D-D rearrangements are commonly detected in CD10+ CD19+ human lymphoid precursor acute lymphoblastic leukemia (ALL) samples, whereas D-J and V-D-J rearrangements are not detected (51, 52). V-D and D-D rearrangements are detected at high frequency in early murine fetal thymocyte hybridomas (8) and in human fetal liver and fetal thymus T cell samples (Fig. 3 C). Finally, we have commonly detected Dα2-Dα3-Jα1 rearrangements as a 2.8-kb XbaI fragment in DNA from human γ/δ T cell clones and polyclonal postnatal thymocytes using a Jα1 probe, but have not observed a 4.8-kb fragment predicted for Dα3-Jα1 rearrangement (16, 46) (data not shown). This high ratio of D-D-J to D-J rearrangements is most consistent with initial D-D joining, and subsequent joining of D-D to J.

Despite these observations, TCR δ-D-J rearrangements are documented to occur in murine fetal and neonatal thymocytes (8, 53). Further, we detected low levels of Dα3-Jα1 rearrangements in human fetal liver and fetal thymus T cell samples by PCR (data not shown). Thus, at the endogenous TCR δ locus, D to J joining may indeed occur on chromosomes that had not previously undergone V-D or D-D rearrangement. The transgenic minilocus may differ from the endogenous locus only in the efficiency of the initial V to D joining event, once it is activated.

We found no evidence for direct joining of V to J, despite the fact that V to J joining should be allowed according to the 12/23 rule. On the contrary, all sequenced junctions showed evidence of Dα3 usage. This result is not due to selection for a functional TCR protein, because mutations were introduced into the V regions to destroy their open reading frames. Thus, Dα3 usage must be dictated by the rearrangement mechanism itself. Early activation of V and D segment accessibility coupled with efficient V-D joining would insure the incorporation of Dα3 in rearranged transgenes.

Dα3 is almost universally found in rearranged human TCR δ genes, as is the homologous Dα2 in rearranged murine TCR δ genes (14, 16, 17, 34, 54). This is also likely to result from ordered rearrangement rather than from selection for a functional TCR, because this is a property of both in-frame and out-of-frame rearranged human and murine TCR δ genes and a property of rearranged TCR δ genes in mutant mice that do not express a TCR γ/δ (18). By contrast, the usage of murine Dα1 and human Dα1 and Dα2 is not universal, and appears to be developmentally regulated (14, 16, 17, 54). Together, these data suggest that human Dα3 and murine Dα2 may be the earliest gene segments to be activated within the endogenous TCR δ loci.

The lineage relationship between α/β and γ/δ T-cell compartments has been the subject of much discussion. It was initially proposed that thymocytes might first activate TCR δ and then TCR α rearrangement in an attempt to generate a functional TCR,
with the outcome of the rearrangement process dictating cell lineage (11). Subsequently, Winoto and Baltimore (36) analyzed extrachromosomal circles that are the products of $V\alpha$ to $J\chi$ rearrangement, and found TCR $\delta$ to be in the germ-line configuration in such products. This result would be consistent with a lineage decision before gene rearrangement that dictates TCR $\delta$ or $\alpha$ rearrangement. In support of this interpretation, other investigators have shown that $\alpha/\beta$ T lymphocyte development can occur in transgenic mice carrying functionally rearranged TCR $\gamma$ and $\delta$ genes (37, 38). However, the analysis of gene rearrangements in murine fetal and neonatal thymocyte hybridomas has clearly shown that TCR $\delta$ and $\alpha$ rearrangements can coexist in individual T cell clones (42), and in one study, rearranged TCR $\delta$ genes were detected in extrachromosomal circular DNA (41).

We found minilocus V-D-J rearrangement to be essentially equivalent in $\alpha/\beta$ and $\gamma/\delta$ T cells. This clearly implies that the cis-acting elements within the minilocus construct that control rearrangement do not respond to signals that direct lineage commitment. This result would be consistent with the view that there is no lineage commitment before gene rearrangement. However, it is also possible that a lineage commitment step could precede and therefore influence rearrangement, but that our minilocus construct does not include the cis-acting regulatory elements that are responsive to the commitment signal. Plausible candidates for these elements might be $\delta$ Rec and pseudo-$J\alpha$ (33, 39, 62). These elements were shown to rearrange in the thymus and mediate deletion of the TCR $\delta$ gene, and the activation of this rearrangement was postulated to be a mechanism whereby thymocytes would commit to the TCR $\alpha/\beta$ lineage. However, evidence to indicate that this represents the major pathway to TCR $\alpha$ gene rearrangement in vivo has been lacking. Our results indicate that without intervention by $\delta$ Rec (or perhaps, TCR $\alpha$ rearrangement), the TCR $\delta$ gene could rearrange in virtually all developing thymocytes. Thus, a temporal competition between $\delta$ Rec (or TCR $\alpha$) and TCR $\delta$ rearrangement within T cells precommitted to the $\alpha/\beta$ lineage might determine the frequency with which TCR $\delta$ rearrangements occur in the $\alpha/\beta$ lineage. Our results are clearly consistent with the detection of TCR $\delta$ and $\alpha$ rearrangements on homologous chromosomes of some T cell clones (42).

The analysis of TCR $\delta$ gene rearrangement in an enhancer-negative minilocus argues that the two steps in the rearrangement pathway, V to D and V-D to J, are controlled by separate regulatory elements. Only V-D to J rearrangement is severely impaired in the absence of the enhancer. It is generally accepted that gene segments are activated to rearrange by modulating their accessibility to components of the recombinase machinery (1, 3). Further, enhancer elements can function to modify regional chromatin accessibility (63, 64). Because V to D rearrangement still occurs in the enhancer-negative minilocus, we infer that the TCR $\delta$ enhancer influences the accessibility of the $J\alpha\beta$ and $J\chi\alpha$ segments within the construct, but does not significantly influence the accessibility of $D\chi\beta$ (see Fig. 10). This result is quite striking, because $J\alpha\beta$ and $D\chi\beta$ are separated by only 0.9 kb, both in the minilocus and in the endogenous TCR $\delta$ locus.

Our results argue that there are probably distinct cis-acting elements within the construct that control access to $D\chi\beta$ (and to $V\delta\gamma$ gene segments). Further, our results imply the presence of a domain boundary between $D\chi\beta$ and $J\chi\alpha$ that limits the TCR $\delta$ enhancer to influence J segment but not D segment accessibility and reciprocally, limits more 5' regulatory elements to influence D segment but not J segment accessibility.

Boundary elements that define independent domains of gene activity that are insulated from adjacent regulatory influences play an important role in gene expression (for a review see references 65, 66). Transgenes flanked by these boundary elements can be insulated from position effects (58-61, 67), promoters flanked by these elements can be insulated from a nearby enhancer (67-69), and mutational loss of boundary elements has been implicated in misregulated gene expression (70). Our data suggest that an insulator that is perhaps analogous to the chicken lysozyme A element, the Drosophila 87A7 ssc element, or the chicken $\beta$-globin 5'HSA, may reside between $D\chi\beta$ and $J\chi\alpha$, and may play an important role in regulating stepwise rearrangement of the TCR $\delta$ gene. Within the endogenous TCR $\alpha/\delta$ locus, this insulator may demarcate the 5' boundary of a regulatory domain in chromatin that is opened by the TCR $\delta$ enhancer during T cell development. We predict the 3' boundary to lie between $C\alpha$ and $J\alpha\beta$ gene segments.

It is of interest to contrast our results with those of Ferrier et al. (31), who studied the role of the lymphoid specific Ig H chain enhancer ($E\mu$) in the rearrangement of a hybrid TCR/Ig $V\beta$-$D\chi\beta$-$J\chi\beta$-$E\mu$-$C\mu$ test construct. In the presence of $E\mu$, construct D-J rearrangement was detected in B and T cells, construct V-D-J rearrangement was detected in T cells, and construct V-D rearrangement was essentially undetectable. In the absence of $E\mu$, all construct rearrangement was ablated. These data were interpreted to indicate that D to J rearrangement is controlled by $E\mu$, whereas V to D-J rearrangement requires, in addition to $E\mu$, tissue-specific regulatory elements associated with $V\delta\gamma$. Because no rearrangement occurred without $E\mu$, the enhancer must in this case con-
control access to both D and J segments. In the TCR δ minilocus, V to D joining is independent of the nearby TCR δ enhancer, arguing that the enhancer controls access to J segments only. The difference may be that the boundary element that we propose to exist between Dα and Jα segments may not be present in the analogous position in the TCR β and IgH loci. In the TCR β and IgH loci, which are characterized by early D-J rearrangement, early activation of Eα or Eγ presumably initiates D-J rearrangement before V segments are available. In the TCR δ locus, which is characterized by early V-D rearrangement, activation of V and D segments presumably occurs before enhancer-dependent activation of J segments. The observation that human lymphoid precursor ALL samples display TCR δ V-D and D-D rearrangements (but not D-J and V-D-J), whereas T-lineage ALL samples display V-D-J rearrangements (51, 52), could indicate that the TCR δ enhancer is activated in the latter, but not the former cell population.

Although an important role for the TCR δ enhancer in targeting rearrangement is clear, the mechanism by which targeting occurs is not. A substantial body of literature has established a tight correlation between the onset of germline transcription and the onset of gene rearrangement, but recent studies argue that transcriptional activity per se may not be causal in driving rearrangement (28, 29, 71). The enhancer could provide a targeting function that is also important for transcriptional activity, such as a local change in chromatin structure (64) or DNA methylation (27, 29). Clearly, it will be valuable to assay such parameters across V, D, and J segments within the enhancer-positive and -negative transgenes in future experiments. In addition, cis elements of the TCR δ enhancer that are essential for transcriptional activation have been identified (72, 73) and this system offers the opportunity to test the role of such elements in targeting gene rearrangement. Finally, it should be possible to use this system to identify additional regulatory elements that mediate Dα and Vs gene segment targeting, and that function to define domain boundaries that restrict the influence of regulatory elements to discrete regions of the locus.

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