Enhancer-dependent and -independent Steps in the Rearrangement of a Human T Cell Receptor δ 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 α/δ locus, we generated transgenic mice that carry a germline human TCR δ minilocus that includes $V_\delta1$, $V_\delta2$, $D_\delta3$, $J_\delta1$, $J_\delta3$, and C_δ segments, and either contains or lacks the TCR δ 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 δ locus. V-D-J rearrangement is T cell specific, but is equivalent in α/β and γ/δ T lymphocytes. Thus, either there is no commitment to the α/β and γ/δ T cell lineages before TCR δ gene rearrangement, or if precommitment occurs, it does not operate directly on TCR δ gene *cis*-acting regulatory elements to control TCR δ 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 $J_\delta1$ but not $D_\delta3$, suggesting that a boundary between two independently regulated domains of the minilocus lies between these elements. Within the endogenous TCR α/δ locus, this boundary may represent the 5′ end of a chromatin regulatory domain that is opened by the TCR δ enhancer during T cell development. The position of this boundary may explain the unique propensity of the TCR δ locus to undergo early V to D rearrangement. Our results indicate that the TCR δ enhancer performs a crucial targeting function to regulate TCR δ 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 κ or λ L chain locus (1). Similarly, T cell development is characterized by the ordered rearrangement and expression of TCR genes (4–6). The TCR δ 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 γ and β loci, but TCR α rearrangement does not begin until fetal day 17 (9–13). Furthermore, in mice and humans, the rearrangement of distinct V_γ and V_δ 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 δ gene mutant mice still undergo stepwise TCR γ and δ gene rearrangement in the absence of surface TCR γ/δ 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-

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petence for rearrangement (24–30). However, Ferrier et al. (31) demonstrated that V-D-J rearrangement of a hybrid TCR β/Ig H chain locus test substrate integrated into the germline of transgenic mice is absolutely dependent on the presence of the Ig H chain enhancer (E_μ) within the construct. This experiment argues that an enhancer can provide a requisite targeting function important for regulating gene rearrangement.

The TCR α and δ genes are encoded within a single complex genetic locus, with TCR δ D, J, and C gene segments located between V_α and J_α gene segments (7, 32–35). This organization dictates the deletion of TCR δ gene segments in cells with V_α to J_α rearrangements. γ/δ T cells typically display rearranged γ, δ, and β genes, but germline TCR α, whereas α/β T cells typically display rearranged α, β, and γ genes, with TCR δ deleted. The decision to rearrange TCR α versus δ is therefore a critical event in determining the fate of developing T cells.

A number of studies have addressed the lineage relationship of γ/δ and α/β T cells. Specifically, these studies have asked whether T cell precursors are precommitted to attempt TCR δ or α rearrangement, but not both (36). Alternatively, T cell precursors might initially attempt TCR δ rearrangement, and failing the expression of a functional TCR, might then attempt TCR α rearrangement (11). Thus, cell lineage could dictate gene rearrangement, or alternatively, gene rearrangement could dictate cell lineage. Because TCR α/β lymphocytes typically display TCR α rearrangements on both chromosomes and have deleted both copies of the TCR δ locus, it is not possible to directly deduce the rearrangement potential of the TCR δ locus in the cells of the α/β lineage. Indirect evidence that has accumulated from the analysis of extrachromosomal circular DNA in thymocytes (36), from transgenic mice that carry rearranged TCR genes (37, 38), from the identification of a specific TCR δ deletional rearrangement in thymocytes (39), and from the identification of lineage-specific silencer elements that regulate TCR α expression (40), all tend to support the notion that T cell precursors may commit to the TCR γ/δ or α/β lineage before gene rearrangement. However, evidence to the contrary also exists, and this tissue has not been resolved conclusively (41, 42).

To begin to explore the control of TCR gene rearrangement during T cell development, we have generated transgenic mice carrying a germline human TCR δ minilocus. We find that this construct rearranges in a stepwise fashion that in many ways mimics the endogenous TCR δ locus. This has allowed us to assess the role of the intronic TCR δ enhancer in targeting the rearrangement process. Further, since the transgene is not embedded within the TCR α locus, we have been able to directly compare the rearrangement of this gene in α/β and γ/δ T lymphocytes, and thus explore the relationship between lineage determination and gene rearrangement at the TCR α/δ locus.

Materials and Methods

Constructs. The transgene $V_\delta1$-$V_\delta2$-$D_\delta3$-$J_\delta1$-$J_\delta3$-E_δ-C_δ 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 XbaI sites were destroyed except for two that flank E_δ. Step 1: A 3.9-kb XbaI fragment containing $D_\delta3$ 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 XbaI-digested pBluescript. Step 2: the 10.5-kb BamHI-KpnI fragment containing $J_\delta3$, E_δ, and C_δ was ligated into BamHI- and KpnI-digested plasmid generated in step 1 to yield $D_\delta3$-$J_\delta3$-E_δ-C_δ. Step 3: The 1.8-kb XbaI-XbaI fragment containing $J_\delta1$ was blunt ended as above and cloned into BamHI-digested, blunt-ended, and phosphatase-treated plasmid from step 2 to yield $D_\delta3$-$J_\delta1$-$J_\delta3$-E_δ-C_δ. Step 4: A 3.1-kb XbaI-SalI fragment containing $V_\delta2$ cloned in pBluescript was linearized within the $V_\delta2$ coding region by digestion with PvuI, treated with T4 polymerase to generate blunt ends, and religated to generate a 2-bp frame shift. Step 5: A 3.1-kb EcoRI fragment containing $V_\delta1$ cloned in pBluescript was linearized within the $V_\delta1$ 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 $V_\delta2$ fragment generated in step 4 was excised from the plasmid by digestion with XbaI and SalI, blunt ended by Klenow treatment, and cloned into XbaI-digested, blunt-ended, and phosphatase-treated $V_\delta1$ plasmid from step 5. Step 7: Insert containing $V_\delta1$ and $V_\delta2$ 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 $V_\delta1$-$V_\delta2$-$J_\delta1$-$D_\delta3$-$J_\delta3$-E_δ-C_δ. Step 8: The enhancer-negative construct was generated from the step 7 plasmid by digestion with XbaI to liberate a 1.4-kb fragment containing E_δ, 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 $V_\delta1$ and $V_\delta2$ 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 C_δ 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 $MgCl_2$, 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 45-s denaturation 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. The $V_\delta1$, $V_\delta2$, $J_\delta1$, and $J_\delta3$ oligonucleotides have been described previously (16). Additional oligonucleotides used were C_δA: ATACCAAACCATCCGTTTTTG; C_δB: ACCTGTAGAATCTGTCTTTCAC; and 5′ of $D_\delta3$: CTCCATGAGACGTTTAAGTACC.

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′$J_\delta1$, a 0.4-kb XbaI-PstI fragment mapping 5′ of $J_\delta1$ that was isolated from the genomic 1.8-kb $J_\delta1$ XbaI fragment; $J_\delta1$, a 1.4-kb PstI-XbaI fragment isolated from the same fragment; and 5′ $D_\delta3$, a 0.5-kb HindIII-PstI fragment isolated from the genomic 3.9-kb XbaI fragment carrying $D_\delta3$. The C_δ, $V_\delta1$, and $V_\delta2$ 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 α/β IgG), GL3 (R-PE-conjugated hamster anti-mouse TCR γ/δ 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), Fc Block (rat anti-mouse FcγII 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 × 10^5 cells were washed three times in 2% BSA and 0.1% sodium azide in PBS and were incubated in 50 μl of the same buffer containing 20 μg/ml of appropriate Ab for 30 min at 4°C. Cells were washed three times and were then resuspended in 50 μl of the same buffer containing the appropriate second step reagent for a 30-min incubation at 4°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′) followed by cell sorting. Thymic α/β and γ/δ T cells were purified by two-color staining of thymocytes (10^7 cells/ml) using biotin-conjugated H57-597 and PE-conjugated GL3 (5 μg/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 δ 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 $V_\delta2^+$ culture of γ/δ 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 δ locus (Fig. 1). These included a 3.1-kb fragment carrying $V_\delta1$, a 3.1-kb fragment carrying $V_\delta2$, a 3.9-kb fragment carrying $D_\delta3$, a 1.8-kb fragment carrying $J_\delta1$, and a 10.5-kb fragment carrying $J_\delta3$ and C_δ (35, 46). The latter fragment also contains the previously characterized TCR δ transcriptional enhancer within the $J_\delta3$-C_δ intron (49). $D_\delta3$ was included in the construct because it is the one human D_δ segment that is almost universally used in rearranged TCR δ genes (16, 17, 34). The $V_\delta1$ and $V_\delta2$ gene segments were both included within the construct because they appear to be differentially activated during human fetal thymic development (16, 17). $V_\delta2$ rearrangements are abundant in very early fetal thymocytes, whereas $V_\delta1$ rearrangements predominate in late fetal and neonatal thymocytes. Further, $V_\delta2$ usage is restricted to γ/δ cells, whereas $V_\delta1$ is used in both γ/δ and α/β 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 $V_\delta1$ and $V_\delta2$ 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 δ enhancer from the

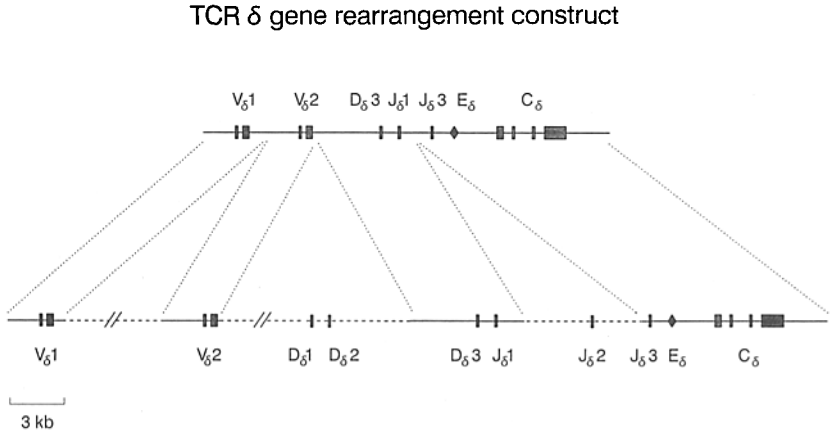


Figure 1. Schematic representation of the human TCR δ minilocus. The transgene (*top*) was constructed from segments of the human TCR δ locus (*bottom*). (*Rectangles*) The exons of V, D, J, and C gene segments. (*Diamond*) The TCR δ transcriptional enhancer. Mutations were introduced into the V gene 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 $J_\delta 3$ and C_δ and flank the enhancer. Finally, we used oligonucleotide primers specific for $V_\delta 1$, $V_\delta 2$, $J_\delta 1$, and $J_\delta 3$ 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 unrearranged 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 $V_\delta 1$, $V_\delta 2$, and C_δ probes. Another, founder F57, carried a minilocus that was disrupted at the 3′ end. Of two EcoRI fragments detected by a C_δ 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 C_δ does not significantly influence construct rearrangement. A third founder, F63, carried a minilocus with a 5′ truncation that deleted the $V_\delta 1$ gene segment, but retained the $V_\delta 2$ gene segment. Founders were mated with C57BL/6 × SJL F_1 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 $V_\delta 1$ and $V_\delta 2$ gene segments for detection. Parallel samples were analyzed by PCR with a pair of C_δ primers, and were detected with a radiolabeled C_δ 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 C_δ 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 $V_\delta 2$-$J_\delta 1$ and $V_\delta 1$-$J_\delta 1$, 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 $V_\delta 1$ or $V_\delta 2$ to $D_\delta 3$. Because $D_\delta 3$ and $J_\delta 1$ are separated by only 0.9 kb in the germline configuration, the combination of a $V_\delta 1$ or $V_\delta 2$ primer with a $J_\delta 1$ primer could amplify a V-D rearranged fragment of 1.2 kb that contains the 0.9-kb genomic segment between $D_\delta 3$ and $J_\delta 1$ (Fig. 3 *B*). This rearrangement would not be detected with a $J_\delta 3$ primer, since the distance between $D_\delta 3$ and $J_\delta 3$ is much greater. To prove that the 1.2-kb fragment indeed represented a V-D rearrangement, Southern blots carrying this fragment were probed with a radiolabeled fragment (5′ $J_\delta 1$) derived from the region between $D_\delta 3$ and $J_\delta 1$. Whereas the $V_\delta 1$ probe hybridized with both the 1.2- and 0.3-kb fragments, the 5′ $J_\delta 1$ probe selectively hybridized with the 1.2-kb fragment, thereby identifying it as a $V_\delta 1$-$D_\delta 3$ rearrangement (Fig. 3 *A*). The partial $V_\delta 1$-$D_\delta 3$ and $V_\delta 2$-$D_\delta 3$ 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 $V_\delta 2$ and $J_\delta 1$ primers, we detected $V_\delta 2$-$D_\delta 3$-$J_\delta 1$ and $V_\delta 2$-$D_\delta 3$ 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 $V_\delta 2$-$D_\delta 3$-$J_\delta 1$ and $V_\delta 2$-$D_\delta 3$ 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 $D_\delta 3$-$J_\delta 1$ rearrangement by PCR using 5′ $D_\delta 3$ and $J_\delta 1$ primers, even though these primers did amplify a 1.2-kb fragment originating from unrearranged chromosomes (data not shown). Furthermore, we did not detect the predicted $D_\delta 3$-$J_\delta 1$ fragment using a $J_\delta 1$ 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 β 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-

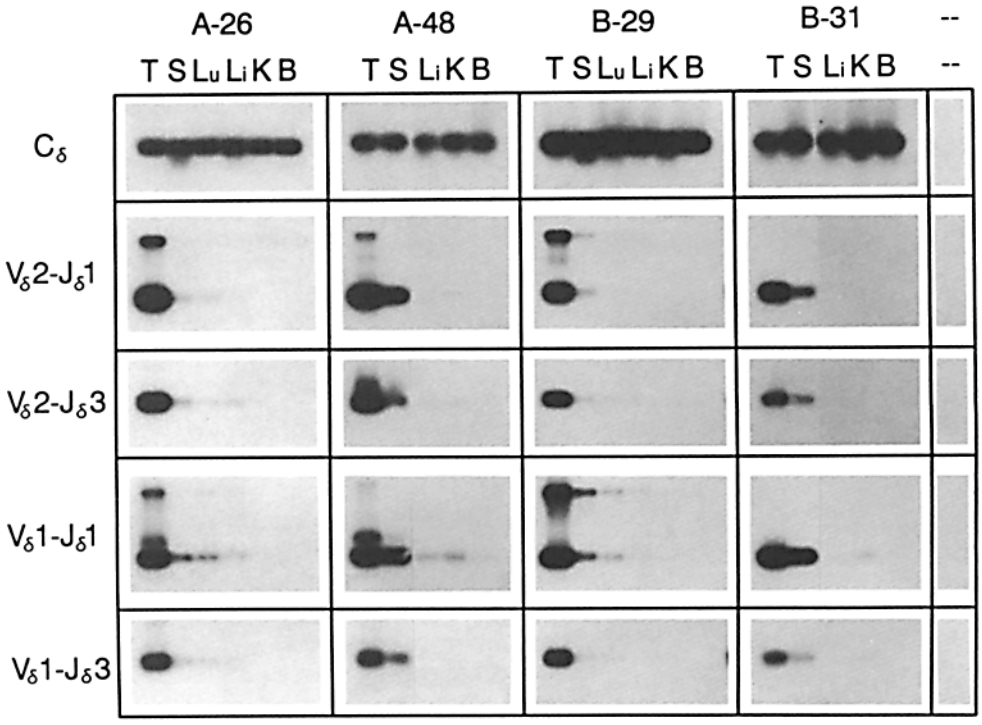
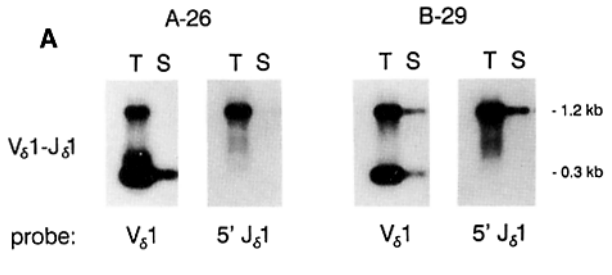
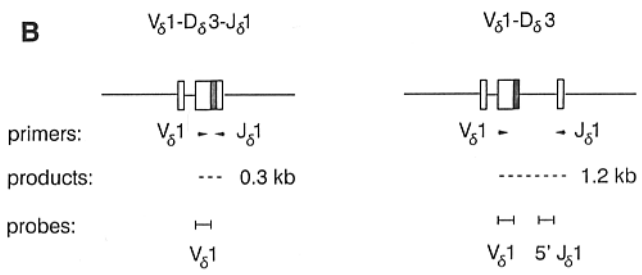


Figure 2. Tissue specificity of TCR δ minilocus rearrangement. The indicated primer pairs were used to detect transgene rearrangements by PCR, using genomic DNA from thymus (*T*), spleen (*S*), lung (*Lu*), liver (*Li*), kidney (*K*), and brain (*B*), or no DNA (- -) as templates. A pair of C_δ primers served as an internal control. The mice analyzed were A-26 (3-wk-old), A-48 (6-wk-old), B-29 (2-wk-old), and B-31 (9-wk-old). Southern blots of PCR products were probed with radiolabeled $V_\delta 2$, $V_\delta 1$, and C_δ cDNA fragments. Autoradiographic exposures were adjusted so that tissue specificity could be assessed in each panel.

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. 4). 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 $V_\delta 1$-$D_\delta 3$-$J_\delta 1$ rearrangements, and about 3% of the transgenes





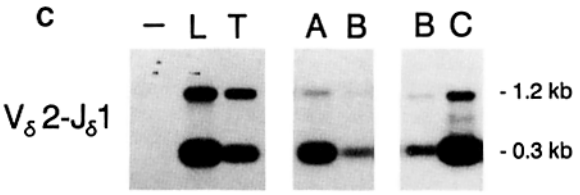


Figure 3. TCR δ minilocus V-D and V-D-J rearrangement. (*A*) Panels from the Southern blot in Fig. 2 (DNA from A-26 and B-29 thymus (*T*) and spleen (*S*) amplified with $V_\delta 1$ and $J_\delta 1$ primers) were developed with radiolabeled $V_\delta 1$ and 5′ $J_\delta 1$ probes. (*B*) The amplification and detection of the 0.3-kb V-D-J and 1.2-kb V-D rearrangements are diagrammed. (*C*) $V_\delta 2$ and $J_\delta 1$ primers were used to amplify V-D and V-D-J rearrangements in DNA samples from human fetal liver (*L*) and fetal thymus (*T*) T cell lines, and from thymuses of mice A-26 (*A*), B-29 (*B*), and C-306 (*C*) (6-wk-old). Rearrangements were detected using a radiolabeled $V_\delta 2$ probe. The panels comparing A-26 to B-29 and B-29 to C-306, are the results of separate experiments. Control C_δ signals were equivalent for the DNA samples analyzed within each panel (data not shown).

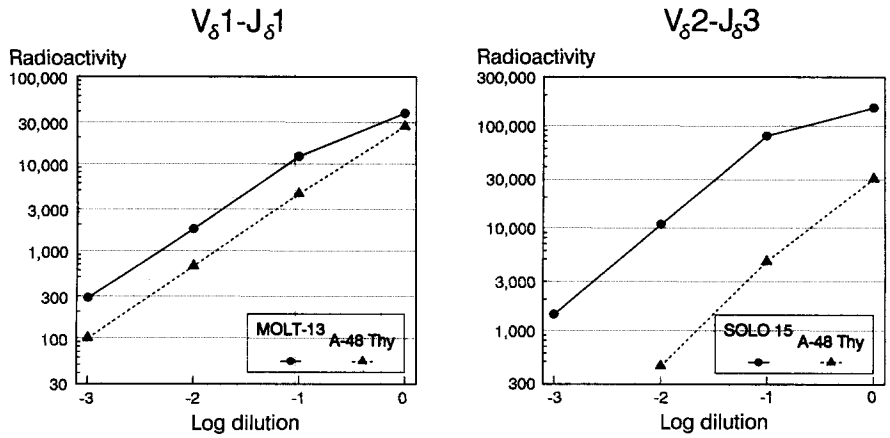


Figure 4. Quantification of TCR δ minilocus V-D-J rearrangement. Serially diluted samples of A-48 thymus DNA, human γ/δ T cell Molt-13 DNA, and human γ/δ T cell Solo 15 DNA were subjected to PCR using the indicated primer pairs and analyzed on Southern blots using Vδ1 and Vδ2 probes. Amplified 0.3-kb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

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-Jδ1 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 Jδ1 probes (Fig. 5). We detected an abundant rearranged fragment of 1.7 kb with both the Vδ1 and Jδ1 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

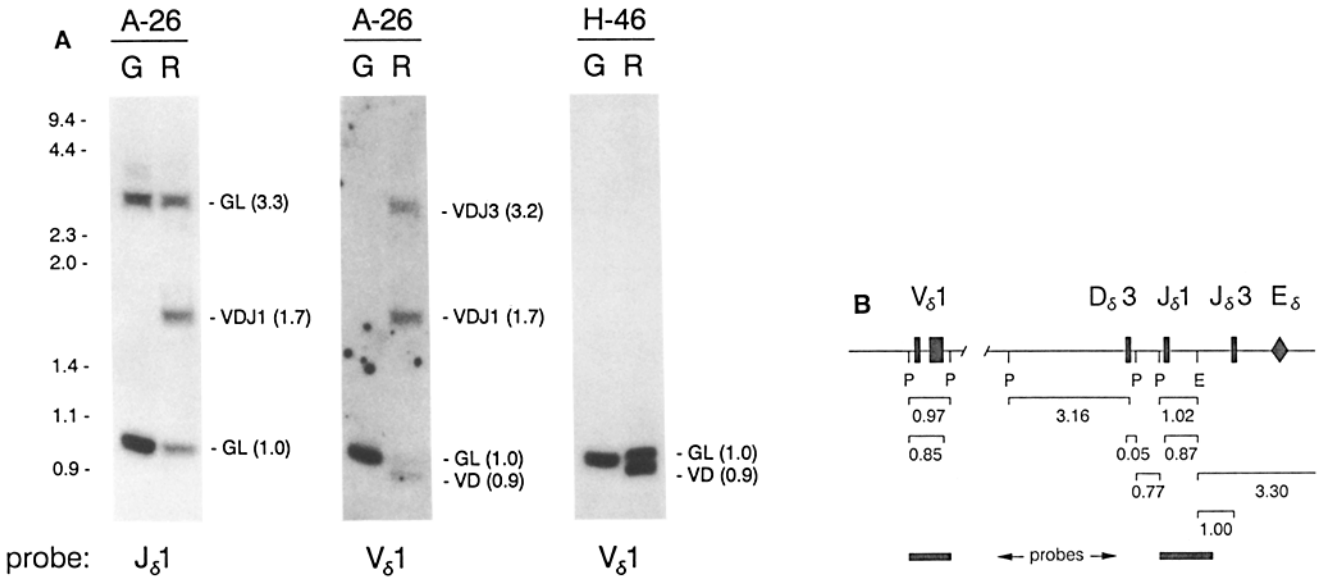


Figure 5. TCR δ minilocus rearrangement analyzed directly on Southern blots. (*A*) PstI-plus EcoRI-digested A-26 tail DNA (germline [*G*]) and thymus DNA (rearranged [*R*]) was analyzed on Southern blots using radiolabeled Jδ1 and Vδ1 probes. Similarly digested H-46 tail DNA (*G*) and thymus DNA (*R*) 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 Jδ1 probe is 2.9 kb. (*B*) Vδ1, Dδ3, Jδ1 and Jδ3 segments, PstI and EcoRI sites, and Vδ1 and Jδ1 probes are mapped in the enhancer-positive minilocus.

	$V_\delta 1/2$	P	N	P	$D_\delta 3$	P	N	P	$J_\delta 1$
GERMLINE:									
1	TTGGGGAACT				ACTGGGGGATACG				ACACCGATAAA
2	CTGTGACACC								
FETAL DAY 17:									
1	TTGG			AGT	ACTGGGGGATAC				ACCGATAAA
1	TTGGGGAACT				ACTGGGGG				ACACCGATAAA
1	TTGGGGAACT				GG				CACCGATAAA
1	TTGGGGAA				GGGG				ACACCGATAAA
2	CTGTGACACC				GGGGG				ACACCGATAAA
2	CTGTGACAC				TGGGGGATAC				ACCGATAAA
2	CTGTGA			T	ACTGGGGG			GT	ACACCGATAAA
2	CTGTGACA			T	ACTGGGGGATAC				ACCGATAAA
NEONATAL A-26:									
1	TTGGGG				ACTGGGGG		CT	T	ACACCGATAAA
1	TTGGG				ACTGGGGGATA		G		CACCGATAAA
1	TTGGGGAA		G		ACTGGGGGATA		GCGAAG	GT	ACACCGATAAA
1	TTGGGGAACT		G		ACTGGGGG		GT	T	ACACCGATAAA
1	TTGGGG			T	ACTGGGGGATACG	CG	GG		CGATAAA
1	TTGGGG		GTTC	T	ACTGGGGG		TT		CACCGATAAA
1	TTGGGGAACT	AG			TGGGGGATACG		TCGG		ACCGATAAA
1	TTGGGGAACT	A	TC		CTGGGGGATAC				CGATAAA
2	CTGTGACACC				TGGGGG		CCCC		ACCGATAAA

Figure 6. Nucleotide sequences of V-D-J rearrangements in fetal and neonatal thymocytes. Junctional sequences of 0.3-kb PCR products amplified using $V_\delta 1$ and $J_\delta 1$ or $V_\delta 2$ and $J_\delta 1$ primers from fetal day 17 and A-26 thymus DNA are compared with germline sequences (*top*, underlined). (*Left*) Numbers indicate V_δ segment used. Palindromic "P" nucleotides and template independent "N" nucleotides are identified.

the signals for the rearranged fragments detected with the $V_\delta 1$ probe, we estimate that ~15% of the $V_\delta 1$ rearrangements in A-26 thymus are partial $V_\delta 1$-$D_\delta 3$ rearrangements. Consistent with the PCR data, partial $D_\delta 3$-$J_\delta 1$ rearrangements of 4.1 kb were not detected.

Fine Structure Analysis of V-D-J Junctions. The recombination signal sequences 3′ of V_δ and D_δ gene segments display 23-bp spacers, whereas those 5′ of D_δ and J_δ 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 δ 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 δ 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 δ V-D-J junctions, and in accord with the known pattern of terminal transferase 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.

Lineage Specificity of TCR δ 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 $V_\delta 1$ and $J_\delta 1$ 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 $V_\delta 1$-$J_\delta 3$, $V_\delta 2$-$J_\delta 1$, and $V_\delta 2$-$J_\delta 3$ 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 α/β and γ/δ T cells, or occurred preferentially in one of these cell populations. A combination of cytotoxic elimination and cell sorting was used

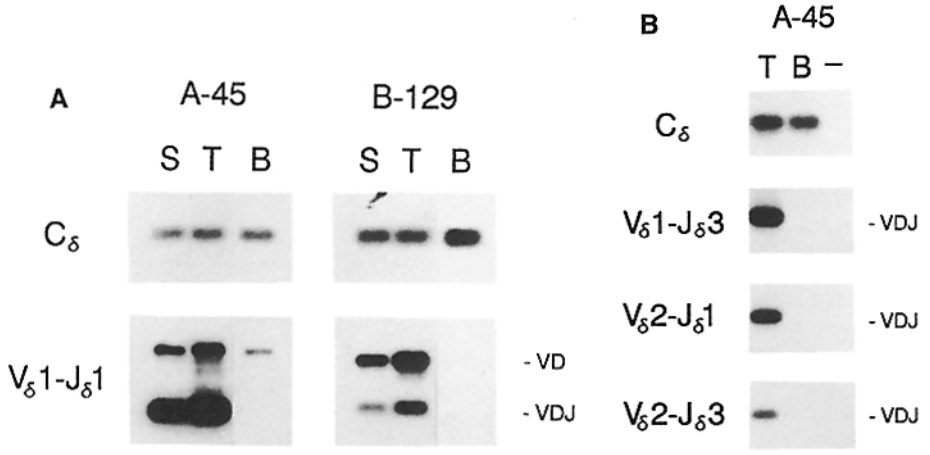


Figure 7. TCR δ minilocus rearrangement in fractionated splenocytes. (*A*) Genomic DNA samples isolated from unseparated splenocytes (*S*), and purified splenic T (*T*) and B (*B*) cells from mice A-45 (25-wk-old) and B-129 (3.5-wk-old) were amplified by PCR with the indicated primers. Southern blots were probed with radiolabeled $V_\delta 1$ and C_δ cDNA fragments. V-D-J and V-D rearrangements are indicated. (*B*) Genomic DNA samples from A-45 T and B populations were amplified by PCR with the indicated primer pairs, and Southern blots were probed with radiolabeled $V_\delta 1$, $V_\delta 2$, and C_δ cDNA fragments.

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 $V_\delta 1$-$D_\delta 3$-$J_\delta 1$ and $V_\delta 1$-$D_\delta 3$-$J_\delta 3$ 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).

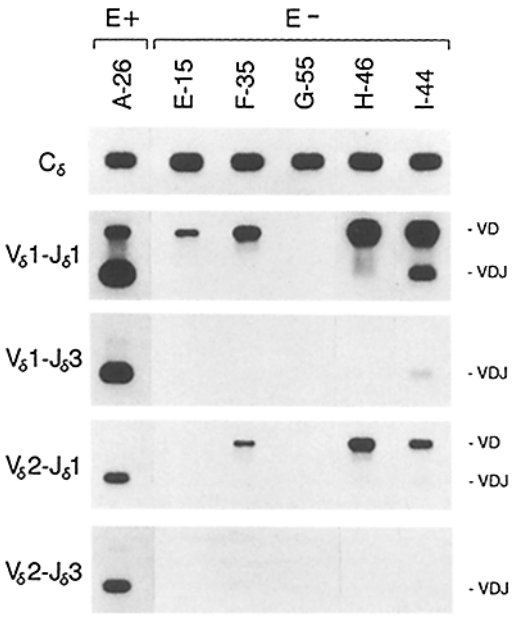


Figure 9. Rearrangement of an enhancer-negative TCR δ minilocus. Thymus DNA from enhancer-positive ($E+$) mouse A-26 and enhancer-negative ($E-$) mice E-15 (2.5-wk-old), F-35 (1.5-wk-old), G55 (2.5-wk-old), H-46 (2.5-wk-old), and I-44 (2.5-wk-old) were amplified by PCR using the indicated primers, and Southern blots were developed using appropriately radiolabeled probes. Probe-specific activities and autoradiographic exposures were similar.

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,

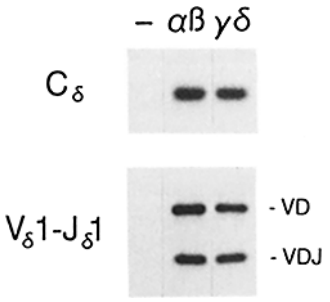


Figure 8. TCR δ minilocus rearrangement in α/β and γ/δ thymocytes. Genomic DNA samples isolated from α/β and γ/δ T cells purified from neonatal thymus (3-d-old), and a no DNA control (−) were amplified by PCR with the indicated primers. Southern blots were probed with radiolabeled $V_\delta 1$ and C_δ fragments. V-D-J and V-D rearrangements are indicated.

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 $V_\delta1$, $V_\delta2$, $D_\delta3$, $J_\delta1$, $J_\delta3$, and C_δ gene segments. 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 $D_\delta2$-$D_\delta3$-$J_\delta1$ rearrangements as a 2.8-kb XbaI fragment in DNA from human γ/δ T cell clones and polyclonal postnatal thymocytes using a $J_\delta1$ probe, but have not observed a 4.8-kb fragment predicted for $D_\delta3$-$J_\delta1$ 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 $D_\delta3$-$J_\delta1$ 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 $D_\delta3$ 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, $D_\delta3$ 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 $D_\delta3$ in rearranged transgenes.

$D_\delta3$ is almost universally found in rearranged human TCR δ genes, as is the homologous $D_\delta2$ 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 $D_\delta1$ and human $D_\delta1$ and $D_\delta2$ is not universal, and appears to be developmentally regulated (14, 16, 17, 54). Together, these data suggest that human $D_\delta3$ and murine $D_\delta2$ 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 cells 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_α to J_α rearrangement, and found TCR δ to be in the germline configuration in such products. This result would be consistent with a lineage decision before gene rearrangement that dictates TCR δ or α rearrangement. In support of this interpretation, other investigators have shown that α/β T lymphocyte development can occur in transgenic mice carrying functionally rearranged TCR γ and δ genes (37, 38). However, the analysis of gene rearrangements in murine fetal and neonatal thymocyte hybridomas has clearly shown that TCR δ and α rearrangements can coexist in individual T cell clones (42), and in one study, rearranged TCR δ genes were detected in extrachromosomal circular DNA (41).

We found minilocus V-D-J rearrangement to be essentially equivalent in α/β and γ/δ 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 δ Rec and pseudo-J_α (33, 39, 62). These elements were shown to rearrange in the thymus and mediate deletion of the TCR δ gene, and the activation of this rearrangement was postulated to be a mechanism whereby thymocytes would commit to the TCR α/β lineage. However, evidence to indicate that this represents the major pathway to TCR α gene rearrangement in vivo has been lacking. Our results indicate that without intervention by δ Rec (or perhaps, TCR α rearrangement), the TCR δ gene could rearrange in virtually all developing thymocytes. Thus, a temporal competition between δ Rec (or TCR α) and TCR δ rearrangement within T cells precommitted to the α/β lineage might determine the frequency with which TCR δ rearrangements occur in the α/β lineage. Our results are clearly consistent with the detection of TCR δ and α rearrangements on homologous chromosomes of some T cell clones (42).

The analysis of TCR δ 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 δ enhancer influences the accessibility of the $J_\delta1$ and $J_\delta3$ segments within the construct, but does not significantly influence the accessibility of $D_\delta3$ (see Fig. 10). This result is quite striking, because $J_\delta1$ and $D_\delta3$ are separated by only 0.9 kb, both in the minilocus and in the endogenous TCR δ locus. Our results argue that there are probably distinct *cis*-acting elements within the construct that control access to $D_\delta3$ (and to V_δ gene segments). Further, our results imply the presence of a domain boundary between $D_\delta3$ and $J_\delta1$ that limits the TCR δ 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 scs element, or the chicken β-globin 5′HS4, may reside between $D_\delta3$ and $J_\delta1$, and may play an important role in regulating stepwise rearrangement of the TCR δ gene. Within the endogenous TCR α/δ locus, this insulator may demarcate the 5′ boundary of a regulatory domain in chromatin that is opened by the TCR δ enhancer during T cell development. We predict the 3′ boundary to lie between C_δ and J_α 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_μ) in the rearrangement of a hybrid TCR/Ig V_β-D_β-J_β-E_μ-C_μ test construct. In the presence of E_μ, 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_μ, all construct rearrangement was ablated. These data were interpreted to indicate that D to J rearrangement is controlled by E_μ, whereas V to D-J rearrangement requires, in addition to E_μ, tissue-specific regulatory elements associated with V_β. Because no rearrangement occurred without E_μ, the enhancer must in this case con-

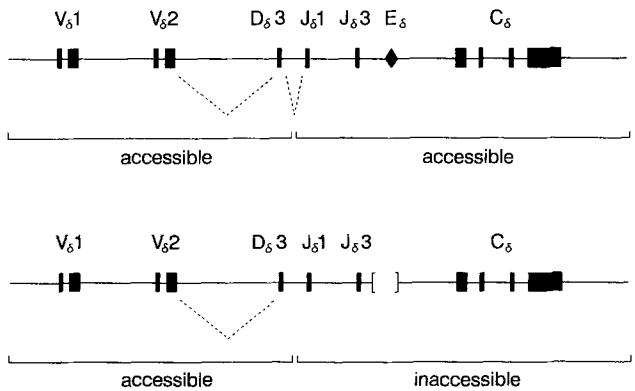


Figure 10. Diagrammatic comparison of the enhancer-positive and -negative TCR δ miniloci. Allowable gene segment rearrangements (- - - -) are interpreted in terms of accessible domains of chromatin.

trol 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 D_δ and J_δ 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 E_β or E_μ 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 D_δ and V_δ 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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References

1. Alt, F.W., T.K. Blackwell, and G.D. Yancopoulos. 1987. Development of the primary antibody repertoire. *Science (Wash. DC).* 238:1079.
2. Lieber, M.R. 1991. Site-specific recombination in the immune system. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 5:2934.
3. Alt, F.W., E.M. Oltz, F. Young, J. Gorman, G. Taccioli, and J. Chen. 1992. VDJ recombination. *Immunol. Today.* 13:306.
4. Fowlkes, B.J., and D.M. Pardoll. 1989. Molecular and cellular events of T cell development. *Adv. Immunol.* 44:207.
5. Strominger, J.L. 1989. Developmental biology of T cell receptors. *Science (Wash. DC).* 244:943.
6. Raulet, D.H., D.M. Spencer, Y.-H. Hsiang, J.P. Goldman, M. Bix, N.S. Liao, M. Zijlstra, R. Jaenisch, and I. Correa. 1991. Control of γδ T-cell development. *Immunol. Rev.* 120:185.
7. Chien, Y.-H., M. Iwashima, K.B. Kaplan, J.F. Elliot, and M.M. Davis. 1987. A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. *Nature (Lond.).* 327:677.
8. Chien, Y.-H., M. Iwashima, D.A. Wettstein, K.B. Kaplan, J.F. Elliot, W. Born, and M.M. Davis. 1987. T-cell receptor δ gene rearrangements in early thymocytes. *Nature (Lond.).* 330:722.
9. Raulet, D.H., R.D. Garman, H. Saito, and S. Tonegawa. 1985. Developmental regulation of T-cell receptor gene expression. *Nature (Lond.).* 314:103.
10. Snodgrass, H.R., Z. Dembic, M. Steinmetz, and H. von Boehmer. 1985. Expression of T-cell antigen receptor genes during fetal development in the thymus. *Nature (Lond.).* 315:232.
11. Pardoll, D.M., B.J. Fowlkes, J.A. Bluestone, A. Kruisbeek, W.L. Maloy, J.E. Coligan, and R.H. Schwartz. 1987. Differential expression of two distinct T-cell receptors during thymocyte development. *Nature (Lond.).* 326:79.
12. Havran, W.L., and J.P. Allison. 1988. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. *Nature (Lond.).* 335:443.
13. Itohara, S., N. Nakanishi, O. Kanagawa, R. Kubo, and S. Tonegawa. 1989. Monoclonal antibodies specific to native murine T-cell receptor γδ: analysis of γδ T cells during thymic ontogeny and in peripheral lymphoid organs. *Proc. Natl. Acad. Sci. USA.* 86:5094.
14. Elliot, J.F., E.P. Rock, P.A. Patten, M.M. Davis, and Y.-H. Chien. 1988. The adult T-cell receptor δ-chain is diverse and distinct from that of fetal thymocytes. *Nature (Lond.).* 331:627.
15. Ito, K., M. Bonneville, Y. Takagaki, N. Nakanishi, O.

Kanagawa, E.G. Krecko, and S. Tonegawa. 1989. Different γδ T-cell receptors are expressed on thymocytes at different stages of development. *Proc. Natl. Acad. Sci. USA.* 86:631.
16. Krangel, M.S., H. Yssel, C. Brocklehurst, and H. Spits. 1990. A distinct wave of human T cell receptor γ/δ lymphocytes in the early fetal thymus: evidence for controlled gene rearrangement and cytokine production. *J. Exp. Med.* 172:847.
17. McVay, L.D., S.R. Carding, K. Bottomly, and A.C. Hayday. 1991. Regulated expression and structure of T cell receptor gamma/δ transcripts in human thymic ontogeny. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:83.
18. Itohara, S., P. Mombaerts, J. Lafaille, J. Iacomini, A. Nelson, A.R. Clarke, M.L. Hooper, A. Farr, and S. Tonegawa. 1993. T cell receptor δ gene mutant mice: independent generation of αβ T cells and programmed rearrangements of γδ TCR genes. *Cell.* 72:337.
19. Schatz, D.G., M.A. Oettinger, and D. Baltimore. 1989. The V(D)J recombination activating gene, RAG-1. *Cell.* 59:1035.
20. Oettinger, M.A., D.G. Schatz, C. Gorka, and D. Baltimore. 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science (Wash. DC).* 248:1517.
21. Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and V.E. Papaionnou. 1992. RAG-1–deficient mice have no mature B and T lymphocytes. *Cell.* 68:869.
22. Shinkai, Y., G. Rathbun, K.-P. Lam, E.M. Oltz, V.E. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A.M. Stall, and F.W. Alt. 1992. RAG-2–deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell.* 68:855.
23. Akira, S., K. Okazaki, and H. Sakano. 1987. Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. *Science (Wash. DC).* 238:1134.
24. Yancopoulos, G., and F. Alt. 1985. Developmentally controlled and tissue-specific expression of unrearranged V_H gene segments. *Cell.* 40:271.
25. Blackwell, T.K., M.W. Moore, G.D. Yancopoulos, H. Suh, S. Lutzker, E. Selsing, and F.W. Alt. 1986. Recombination between immunoglobulin variable region gene segments is enhanced by transcription. *Nature (Lond.).* 324:585.
26. Schlissel, M.S., and D. Baltimore. 1989. Activation of immunoglobulin kappa gene rearrangement correlates with induction of germline kappa gene transcription. *Cell.* 58:1001.
27. Engler, P., D. Haasch, C.A. Pinkert, L. Doglio, M. Glymour, R. Brinster, and U. Storb. 1991. A strain specific modifier on mouse chromosome 4 controls the methylation of independent transgene loci. *Cell.* 65:939.
28. Hsieh, C.-L., R.P. McCloskey, and M.R. Lieber. 1992. V(D)J recombination on minichromosomes is not affected by transcription. *J. Biol. Chem.* 267:15613.
29. Hsieh, C.-L., and M.R. Lieber. 1992. CpG methylated minichromosomes become inaccessible for V(D)J recombination after undergoing replication. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:315.
30. Goldman, J.P., D.M. Spencer, and D.H. Raulet. 1993. Ordered rearrangement of variable region genes of the T cell receptor γ locus correlates with transcription of the unrearranged genes. *J. Exp. Med.* 177:729.
31. Ferrier, P., B. Krippl, T.K. Blackwell, A.J.W. Furley, H. Suh, A. Winoto, W.D. Cook, L. Hood, F. Costantini, and F.W. Alt. 1990. Separate elements control DJ and VDJ rearrangement in a transgenic recombination substrate. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:117.
32. Hata, S., M.B. Brenner, and M.S. Krangel. 1987. Identification of putative human T-cell receptor δ complementary DNA clones. *Science (Wash. DC).* 238:678.
33. Hockett, R.D., J.-P. de Villartay, K. Pollock, D.G. Poplack, D.I. Cohen, and S.J. Korsmeyer. 1988. Human T-cell antigen receptor (TCR) δ-chain locus and elements responsible for its deletion are within the TCR α-chain locus. *Proc. Natl. Acad. Sci. USA.* 85:9694.
34. Loh, E.Y., S. Cwirla, A.T. Serafini, J.H. Phillips, and L.L. Lanier. 1988. Human T-cell-receptor δ chain: genomic organization, diversity, and expression in populations of cells. *Proc. Natl. Acad. Sci. USA.* 85:9714.
35. Satyanarayana, K., S. Hata, P. Devlin, M.G. Roncarolo, J.E. de Vries, H. Spits, J.L. Strominger, and M.S. Krangel. 1988. Genomic organization of the human T-cell antigen-receptor α/δ locus. *Proc. Natl. Acad. Sci. USA.* 85:8166.
36. Winoto, A., and D. Baltimore. 1989. Separate lineages of T cells expressing the αβ and γδ receptors. *Nature (Lond.).* 338:430.
37. Dent, A.L., L.A. Matis, F. Hooshmand, S.M. Widacki, J.A. Bluestone, and S.M. Hedrick. 1990. Self-reactive γδ T cells are eliminated in the thymus. *Nature (Lond.).* 343:714.
38. Ishida, I., S. Verbeek, M. Bonneville, S. Itohara, A. Berns, and S. Tonegawa. 1990. T-cell receptor γδ and γ transgenic mice suggest a role of a γ gene silencer in the generation of αβ T cells. *Proc. Natl. Acad. Sci. USA.* 87:3067.
39. de Villartay, J.-P., R.D. Hockett, D. Coran, S.J. Korsmeyer, and D.I. Cohen. 1988. Deletion of the human T-cell receptor δ-gene by a site-specific recombination. *Nature (Lond.).* 335:170.
40. Winoto, A., and D. Baltimore. 1989. αβ lineage-specific expression of the α T cell receptor gene by nearby silencers. *Cell.* 59:649.
41. Takashita, S., M. Toda, and H. Yamagishi. 1989. Excision products of the T cell receptor gene support a progressive rearrangement model of the α/δ locus. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3261.
42. Thompson, S.D., J. Pelkonen, and J.L. Hurwitz. 1990. Concomitant T-cell receptor α and δ gene rearrangements in individual T-cell precursors. *Proc. Natl. Acad. Sci. USA.* 87:5583.
43. Hogan, B., F. Costantini, and E. Lacy. 1986. Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 332 pp.
44. Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Elrich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Wash. DC).* 239:487.
45. Wigler, M., R. Sweet, G.K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel. 1979. Transformation of mammalian cells with genes from procaryotes and eucaryotes. *Cell.* 16:777.
46. Hata, S., M. Clabby, P. Devlin, H. Spits, J.E. de Vries, and M.S. Krangel. 1989. Diversity and organization of human T cell receptor δ variable gene segments. *J. Exp. Med.* 169:41.
47. Feinberg, A., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6.
48. Coligan, J.E., A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober. 1991. Current Protocols in Immunology. John Wiley & Sons, New York.
49. Redondo, J.M., S. Hata, C. Brocklehurst, and M.S. Krangel. 1990. A T cell specific transcriptional enhancer within the human T cell receptor δ locus. *Science (Wash. DC).* 247:1225.
50. Miossec, C., F. Faure, L. Ferradini, S. Roman-Roman, S. Jitsukawa, S. Ferrini, A. Moretta, F. Treibel, and T. Hercend.

1990. Further analysis of the T cell receptor γ/δ^+ peripheral lymphocyte subset. The $V_\delta 1$ gene segment is expressed with either C_α or C_δ. *J. Exp. Med.* 171:1171.
51. Griesinger, F., J.M. Greenberg, and J.H. Kersey. 1989. T cell receptor gamma and delta rearrangements in hematologic malignancies. Relationship to lymphoid differentiation. *J. Clin. Invest.* 84:506.
52. Biondi, A., P.F. di Celle, V. Rossi, G. Casorati, G. Matullo, G. Giudici, R. Foa, and N. Migone. 1990. High prevalence of T-cell receptor $V_\delta 2$-(D)-$D_\delta 3$ or $D_\delta 1/2$-$D_\delta 3$ rearrangements in B-precursor acute lymphoblastic leukemias. *Blood.* 75:1834.
53. Roth, D.B., P.B. Nakajima, J.P. Menetski, M.J. Bosma, and M. Gellert. 1992. V(D)J recombination in mouse thymocytes: double-strand breaks near T cell receptor δ rearrangement signals. *Cell.* 69:41.
54. Lafaille, J.J., A. DeCloux, M. Bonneville, Y. Takagaki, and S. Tonegawa. 1989. Junctional sequences of T cell receptor $\gamma\delta$ genes: implications for $\gamma\delta$ T cell lineages and for a novel intermediate of V-(D)-J joining. *Cell.* 59:859.
55. Landau, N.R., D.G. Schatz, M. Rosa, and D. Baltimore. 1987. Increased frequency of N-region insertion in a murine pre-B-cell line infected with a terminal deoxynucleotidyl transferase retroviral expression vector. *Mol. Cell. Biol.* 7:3237.
56. Bogue, M., S. Gilfillan, C. Benoist, and D. Mathis. 1992. Regulation of N-region diversity in antigen receptors through thymocyte differentiation and thymus ontogeny. *Proc. Natl. Acad. Sci. USA.* 89:11011.
57. Roth, D.B., J.P. Menetski, P.B. Nakajima, M.J. Bosma, and M. Gellert. 1992. V(D)J recombination: broken DNA molecules with covalently sealed (hairpin) coding ends in scid mouse thymocytes. *Cell.* 70:983.
58. Grosveld, F., G.B. van Assendelft, D.R. Greaves, and G. Kollias. 1987. Position-independent, high-level expression of the human β-globin gene in transgenic mice. *Cell.* 51:975.
59. Bonifer, C., M. Vidal, F. Grosveld, and A.E. Sippel. 1990. Tissue specific and position independent expression of the complete gene domain for chicken lysozyme in transgenic mice. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2843.
60. Kellum, R., and P. Schedl. 1991. A position-effect assay for boundaries of higher order chromosomal domains. *Cell.* 64:941.
61. McKnight, R.A., A. Shamay, L. Sankaran, R.J. Wall, and L. Hennighausen. 1992. Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice. *Proc. Natl. Acad. Sci. USA.* 89:6943.
62. de Villartay, J.-P., D. Mossalay, R. de Chasseval, A. Dalioul, and P. Debre. 1991. The differentiation of human prothymocytes along the TCR-α/β pathway in vitro is accompanied by the site-specific deletion of the TCR-δ locus. *Int. Immunol.* 3:1301.
63. Gross, D.S., and W.T. Garrard. 1988. Nuclease hypersensitive sites in chromatin. *Annu. Rev. Biochem.* 57:159.
64. Pikaart, M., J. Feng, and B. Villeponteau. 1992. The polyoma enhancer activates chromatin accessibility on integration into the HPRT gene. *Mol. Cell. Biol.* 12:5786.
65. Townes, T.M., and R.R. Behringer. 1990. Human globin locus activating region (LAR): role in temporal regulation. *Trends Genet.* 6:219.
66. Eissenberg, J.C., and S.C.R. Elgin. 1991. Boundary functions in the control of gene expression. *Trends Genet.* 7:335.
67. Chung, J.H., M. Whitely, and G. Felsenfeld. 1993. A 5′ element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in Drosophila. *Cell.* 74:505.
68. Stief, A., D.M. Winter, W.H. Stratling, and A.E. Sippel. 1989. A nuclear DNA attachment element mediates elevated and position-independent gene activity. *Nature (Lond.).* 341:343.
69. Kellum, R., and P. Schedl. 1992. A group of scs elements function as domain boundaries in an enhancer-blocking assay. *Mol. Cell. Biol.* 12:2424.
70. Gyurkovics, H., J. Gausz, J. Kummer, and F. Karch. 1990. A new homeotic mutation in the Drosophila bithorax complex removes a boundary separating two domains of regulation. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2579.
71. Engler, P., P. Roth, J.Y. Kim, and U. Storb. 1991. Factors affecting the rearrangement efficiency of an Ig test gene. *J. Immunol.* 146:2826.
72. Redondo, J.M., J.L. Pfohl, and M.S. Krangel. 1991. Identification of an essential site for transcriptional activation within the human T cell receptor δ enhancer. *Mol. Cell. Biol.* 11:5671.
73. Hernandez-Munain, C., and M.S. Krangel. 1994. Regulation of the T-cell receptor δ enhancer by functional cooperation between c-Myb and core-binding factors. *Mol. Cell. Biol.* In press.