Developmentally Programmed Rearrangement of T Cell Receptor V\textsubscript{\textgreek{\textalpha}} Genes Is Controlled by Sequences Immediately Upstream of the V\textsubscript{\textgreek{\textalpha}} Genes

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Summary

Distinct subsets of γδ T cells expressing different V\textsubscript{\textgreek{\textalpha}} and Vδ chains arise in ordered waves during thymic development. In the murine J\textsubscript{γ1-C\textsubscript{γ1}} cluster, the V\textsubscript{2} gene segment is utilized earliest in fetal thymic development, in progenitors of dendritic epidermal T cells (DECs). The V\textsubscript{2} gene segment predominates in the late fetal stages and beyond, in cells destined for the secondary lymphoid organs. Using transgenic TCR\textsubscript{γδ} recombination substrates, we demonstrate that this restricted V\textsubscript{\textgreek{\textalpha}} gene usage is determined by developmentally targeted gene rearrangement. We show that sequences immediately upstream of the V\textsubscript{2} and V\textsubscript{3} genes direct the rearrangement pattern in adult thymocytes. Thus, the choice of V\textsubscript{\textgreek{\textalpha}} genes for recombination is coordinated with distinct differentiation programs in γδ subsets.

Introduction

B and T cell receptor genes are assembled in progenitor lymphocytes by the process of V(D)J recombination. The RAG recombinase targets conserved recombination signal sequences (RSS) that flank the recombining gene segments (Schatz et al., 1992; Sleckman et al., 1996). Although a common recombinase mediates rearrangement of all of the receptor genes, V(D)J rearrangement is developmentally regulated in that progenitor cells undergo rearrangement of different genes in a lineage and developmental stage-specific manner. For instance, complete V(D)J rearrangement of the T and B cell receptor genes is restricted to the appropriate cell type. In T cells, rearrangement of TCR\textsubscript{δ\textgreek{\textalpha}} and V\textsubscript{\textgreek{\textalpha}}\textsubscript{\textdelta} precedes TCR\textsubscript{\textalpha} rearrangement, while in B cells, IgH rearrangement occurs at an earlier developmental stage than Igk or λ\textsubscript{\textalpha}. In the case of IgH and TCR\textsubscript{\textbeta} genes, D to J rearrangement precedes V to DJ rearrangement.

A special case of developmentally regulated V(D)J rearrangement concerns the timing of rearrangement of different V gene segments in a given locus. Developmental regulation of V gene rearrangement impacts the functional repertoire of receptor specificities. At the IgH locus, V genes in the J\textsubscript{\textalpha}-proximal 7183 gene family rearrange preferentially in immature B cells of both fetal and adult origin (Yancopoulos et al., 1984; Malynn et al., 1990; Marshall et al., 1996). Utilization of murine V\textsubscript{\textgreek{\textalpha}} genes is even more strikingly regulated. A developmental switch in the late fetal stage results in the predominant rearrangement of a distinct set of V\textsubscript{\textgreek{\textalpha}} genes in the adult thymus as compared to the early fetal thymus (Raulet et al., 1991). Vδ gene utilization is similarly regulated (Elliott et al., 1988). The Cy1 cluster of the TCR\textsubscript{γδ} locus contains four closely linked but distantly related variable region genes in the following order: V\textsubscript{γ5}-V\textsubscript{γ2}-V\textsubscript{γ4}-V\textsubscript{γ3}, with V\textsubscript{γ3} being the most J\textsubscript{γ1}-proximal. V\textsubscript{γ3} and V\textsubscript{γ4} rearrangements are most prevalent in the early fetal thymus and are very rare in the adult thymus, whereas V\textsubscript{γ2} and V\textsubscript{γ5} rearrangements exhibit the opposite pattern (Garman et al., 1986; Goldman et al., 1993). The rearrangement pattern is reflected by the appearance of distinct sets of γδ cells during ontogeny. V\textsubscript{γ3}\textsubscript{γδ} T cells appear at the earliest stages of thymocyte development, around day 13 of gestation (E13), and disappear from the thymus by E18 (Havran and Allison, 1988). Evidence suggests that V\textsubscript{γ4}\textsubscript{γδ} cells are also only present in the fetal thymus (Itohara et al., 1989). V\textsubscript{γ2}\textsubscript{γδ} cells, and probably V\textsubscript{γ5}\textsubscript{γδ} cells as well, exhibit the opposite developmental pattern, as they first appear only late in fetal thymic development and come to represent a major thymic γδ cell population in the adult (Raulet et al., 1991).

Cells expressing different V\textsubscript{\textgreek{\textalpha}} genes generally exhibit distinct functional characteristics. The V\textsubscript{γ3}\textsubscript{γδ} subset homes specifically to epidermal epithelial tissues, where essentially all of the resident T cells, called dendritic epidermal T cells (DECs), are descendants of early fetal thymic V\textsubscript{γ3}\textsubscript{γδ} cells (Allison and Havran, 1991). DEC cells have the capacity to secrete keratinocyte growth factor, a property most other γδ T cells lack (Boismenu and Havran, 1994). The V\textsubscript{γ4}\textsubscript{γδ} subset migrates preferentially to the lining of the female reproductive tract and the tongue (Itohara et al., 1990). Due to the utilization of a single δ chain sequence by V\textsubscript{γ3}\textsubscript{γδ} and V\textsubscript{γ4}\textsubscript{γδ} cells and the absence of V(D)J junctional diversity in both γ and δ chains, each population expresses only a single receptor specificity (Asamow et al., 1988, 1989; Itohara et al., 1990). On this basis, it has been proposed that these epithelial-associated γδ cells eliminate damaged cells that express common stress-induced self antigens, while at the same time promoting the growth or differentiation of new epithelial cells (Allison and Havran, 1991). In contrast to the localization of the V\textsubscript{γ3}\textsubscript{γδ} and V\textsubscript{γ4}\textsubscript{γδ} subsets, V\textsubscript{γ2}\textsubscript{γδ} T cells are preferentially localized to the secondary lymphoid tissue, where they represent a substantial fraction of circulating γδ cells in most mouse strains (Sperling et al., 1992). Since the diversity of the associated δ chains as well as V(D)J junctional diversity are both much greater in the V\textsubscript{γ2} subset, it has been proposed that these cells recognize foreign antigens (Raulet, 1989). The different properties and localization of the V\textsubscript{\textgreek{\textalpha}}-defined populations suggest that they represent functionally distinct γδ lineages with distinct homing receptors.

Several models have been considered to explain the developmental appearance of the γδ subsets. In one model, the γδ subsets arise from distinct precursors in

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which the Vγ genes are differentially targeted for rearrangement. A second model proposes that the different Vγ-bearing cells arise more or less randomly and that stage-specific thymic ligands promote the survival and/or differentiation of different Vγ-bearing cells at different times in development. While selection may play a role in shaping the γδ repertoire, several lines of evidence suggest that rearrangement of the Vγ genes is not random but rather is developmentally programmed. First, the rearrangement status of the Vγ2, Vγ4, and Vγ3 genes in thymocytes at different stages of development correlates well with the appearance of the different subsets (Garman et al., 1986; Goldman et al., 1993). Almost all of the Vγ3- or Vγ2- hybridomas that have been analyzed in which both γ gene alleles are rearranged contain the same Vγ gene rearrangement at the nonfunctional allele as at the functional allele (Rautel et al., 1991; Heyborne et al., 1993). Second, at the Cγ1 cluster, there is a strong correlation between the timing of germinal transcription of Vγ genes and the timing of the appearance of cells expressing the corresponding Vγ genes (Goldman et al., 1993). A variety of experiments indicate that germinal transcription is somehow related to the accessibility of gene segments to the recombinese (Sleckman et al., 1996). Finally, mice that lack a functional δ gene still display the same pattern of Vγ rearrangement as normal mice, indicating that selection mediated by the γδ receptor is not a prerequisite for the developmentally ordered pattern of γ rearrangements (Itohara et al., 1993). However, it is still formally possible that γ could be selected independently from δ, either alone or associated with a partner chain other than δ. Therefore, while there is substantial evidence that programmed rearrangement plays a role in the developmental appearance of cells expressing distinct Vγ genes, formal proof is still lacking.

How the Vγ genes may be differentially targeted for rearrangement is an intriguing question. For recombination to occur, the chromatin structure surrounding the recombining gene segments must be in a configuration that is "open" and accessible to the recombinesase. It has been proposed that the lineage-specificity and stage-specificity of V(D)J rearrangement at each antigen receptor gene in B and T cells is regulated at the level of transgenic founders were obtained with the specificity of V(D)J recombination at each antigen receptor gene and the timing of the appearance of cells expressing the corresponding Vγ genes (Goldman et al., 1993). A variety of experiments indicate that germinal transcription is somehow related to the accessibility of gene segments to the recombinese (Sleckman et al., 1996). Finally, mice that lack a functional δ gene still display the same pattern of Vγ rearrangement as normal mice, indicating that selection mediated by the γδ receptor is not a prerequisite for the developmentally ordered pattern of γ rearrangements (Itohara et al., 1993). However, it is still formally possible that γ could be selected independently from δ, either alone or associated with a partner chain other than δ. Therefore, while there is substantial evidence that programmed rearrangement plays a role in the developmental appearance of cells expressing distinct Vγ genes, formal proof is still lacking.

The coding region of each Vγ gene in these constructs was mutated by the addition of a restriction enzyme linker, which allows transgene rearrangements to be distinguished from endogenous rearrangements. These mutations also disrupt the reading frames of the genes, resulting in genes that are unable to encode functional proteins. Thus, recombination can be measured independently from any form of cellular selection that could take place if the proteins were expressed. Seven transgenic founders were obtained with the γA construct and six with the γB construct. Most founders were bred to generate transgenic lines in which the offspring were analyzed; however, several of the founders were analyzed directly.

To measure the levels of rearrangement, we employed either a semiquantitative PCR assay or, in some cases, genomic Southern blotting. For the semiquantitative PCR, serial dilutions of DNA from various cell populations, measured in cell equivalents, were amplified with a sense primer specific for either the leader or coding sequence of each Vγ gene and an antisense primer specific for γ1 (Figure 1A). These primers coamplify rearranged DNA from both the endogenous gene and the transgene, but the products could be distinguished by unique restriction enzyme sites present within the transgene. The samples were normalized for DNA content with a β-tubulin PCR and quantitated by comparison to cell lines containing known numbers of Vγ rearrangements (see Experimental Procedures). Initially,
Programmed Rearrangement of TCR Vγ Genes

Figure 1. Rearrangement of TCR Vγ Gene Transgenes
(A and B) Schematic structures of (A) γA and γB and (B) γSW are depicted. The 3′ enhancer (3′ECγ) is denoted by an oval. The asterisks represent the novel restriction site linkers introduced into the Vγ region exons that disrupted the reading frame. In the blowups, genomic Vγ-2 sequences are indicated in black and Vγ-3 sequences in white, with the exchanged regions in γSW indicated in (B). Restriction enzymes are S, SpeI; H, HindIII; and R, EcoRV. All four sites are destroyed in γSW. Arrows indicate the location of PCR primers that were used to detect rearranged DNA. The identity of the PCR primers is as follows: L2 (1); Jγ1 (2); L3 (3); PSVγ2 (4); PSJγ1 (5); PSVγ3 (6).

(C) Semiquantitative PCR analysis of DEC and adult DN thymocyte (THY) DNA from the γA(14) line, which contains a single transgene copy. The primers used were the L3/J1 (Vγ3), L2/J1 (Vγ2), and 5′ and 3′ β tubulin (TUB). The PCR products were digested with the transgene-specific restriction enzymes (Vγ3-EcoRI and Vγ2-NruI). The DNA is measured in cell equivalents, as indicated above the lanes. With respect to the DECs, the DNA for the Vγ-3 and Vγ-2 PCRs was measured in DEC cell equivalents, while the DNA for the β tubulin PCR was measured in total epidermal cell equivalents (see Experimental Procedures). The Vγ3 standard (V3 ST) is the WRD.34 cell line that contains two copies of rearranged Vγ3, and the Vγ2 standard (V2 ST) is the hybridoma DN2.3 that contains two copies of rearranged Vγ2. Endogenous and transgene bands are noted as E and Tg, respectively.

Programmed Rearrangement Is Recapitulated in Transgenes

Initial PCR analysis of a transgenic line that contained a single copy of the γA construct (line 14) demonstrated that the Vγ3 gene in the transgenic construct was rearranged 13 times more frequently in DEC cells than in adult DN thymocytes (Figure 1C). Rearrangements of the endogenous Vγ3 gene exhibited a similar, though somewhat greater (55-fold) bias. In contrast, the Vγ2...
gene in the transgenic construct exhibited the opposite pattern, as it was rearranged more than five times as often in adult thymocytes as in DECs. The endogenous \( V_2 \) gene exhibited a nearly identical preference for rearrangement in adult thymocytes over DECs, although the levels of rearrangement were somewhat higher than for the transgene in both cell types.

Southern blotting of genomic DNA from unfractionated adult thymocytes confirmed the PCR results. Although most adult thymocytes are \( \mu \) lineage cells, these cells usually harbor rearrangements at the \( J_{\gamma 1}-C_{\gamma 1} \) locus, most of which are \( V_2 \) rearrangements. Therefore, total thymocytes and thymic DN cells exhibit a similar pattern of TCR\( \gamma \) rearrangements (Garman et al., 1986). Lane 2 in Figure 2 corresponds to the \( \gamma A(14) \) line harboring one transgene copy (in the figures, transgene copy number is indicated by brackets). Transgene \( V_2 \) rearrangements were observed in total thymocytes, whereas \( V_3 \) rearrangements were undetectable (Figure 2, lane 2). Similarly, in the 15-copy \( \gamma B(3) \) line, a strong band indicating transgene \( V_2 \) rearrangements was evident, but no transgene \( V_3 \) rearrangements were detectable (Figure 2, lane 4). Hence, the added 3' sequences in the \( \gamma A \) construct compared to the \( \gamma B \) construct apparently did not influence the rearrangement pattern, as was also evident in the more extensive PCR analysis presented below. The Southern blot data suggest that the preference for \( V_2 \) rearrangements over \( V_3 \) rearrangements in the adult thymus is even greater than suggested by the PCR results (compare to Figure 3C below). The stronger signal for \( V_2 \) rearrangements evident in the \( \gamma B(3) \) transgenic mice versus the \( \gamma A(14) \) transgenic mice presumably reflects the higher number of transgene copies available to undergo rearrangement in the \( \gamma B(3) \) line.

To further address the relationship between rearrangement levels and transgene copy number, the semiquantitative PCR assay was applied to adult DN thymocytes and DECs for each of the \( \gamma A \) and \( \gamma B \) transgenic lines. The calculated levels of rearrangements per cell were plotted against the transgene copy number (Figures 3A and 3B). Each unit on the y-axis in the graphs corresponds to the rearrangement level of one endogenous \( V_2 \) gene in adult thymocytes (Figure 3A) or one endogenous \( V_3 \) gene in DECs (Figure 3B). The graphs revealed...
Table 1. Rearrangements of Vγ2 and Vγ3 Genes in Fetal Thymocytes

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<th>DAY15 FETAL THYMUS</th>
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<td>V3^a</td>
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<td>0.00150</td>
</tr>
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</table>

^a The V2 and V3 columns refer to the rearrangement/gene copy from PCR data.
^b The V2/V3 columns represent the ratio of V2 to V3 rearrangements/gene copy.

The average means for the rearrangement/gene copy and V2/V3 ratios for the lines derived from each construct are noted. ND = not done.

A correlation between the level of rearrangement and the number of transgene copies. Furthermore, transgene Vγ2 rearrangements were always more prevalent than Vγ3 rearrangements in adult DN thymocytes, and transgene Vγ3 rearrangement levels were always more prevalent than Vγ2 rearrangements in DEC. Since transgene rearrangement levels were roughly proportional to transgene copy number, we subsequently expressed these data as rearrangements per gene copy (Figures 3C and 3D). The data demonstrate that the transgene Vγ genes in different founder lines consistently undergo programmed rearrangement in a manner qualitatively similar to the endogenous γ genes. Since the transgenes could not encode a γ protein and therefore should not confer a selective advantage or disadvantage to the cells that rearranged them, these results provide direct evidence that Vγ gene rearrangement is differentially targeted in the progenitors of DEC versus adult DN thymocytes.

DECs arise from early fetal thymocytes. Therefore, we investigated the pattern of transgene Vγ gene rearrangement in early fetal thymocytes to determine whether it is similar to the pattern in DEC. Indeed, analysis of E14 and E15 fetal thymocytes from several lines demonstrated a consistent bias for Vγ3 over Vγ2 rearrangements, corroborating the DEC data and supporting the assertion that DEC were a relevant cell population in which to study rearrangements that occurred in early fetal thymocytes (Table 1; Figure 4). The absolute levels of rearrangement were lower in fetal thymocytes than in DEC, but this was also true for the endogenous Vγ genes (compare Table 1 and Figure 3D) and presumably reflects the fact that many fetal thymocytes have not yet undergone rearrangement.

**Gene Rearrangement Is Controlled by Sequences Immediately Upstream of the Vγ2 and Vγ3 Genes**

Since the γA and γB transgenes rearranged their gene segments in a developmentally ordered fashion, we were able to manipulate sequences within these constructs in an effort to identify cis-acting elements that specify the developmental pattern of rearrangement. To assess whether regulatory sequences upstream of the Vγ2 and Vγ3 genes play a role in targeting the respective Vγ genes for recombination, a transgene recombination substrate was constructed in which these regions of the number of transgene copies. Furthermore, transgene Vγ3 rearrangements were always more prevalent than Vγ2 rearrangements in fetal thymocytes, and transgene Vγ3 rearrangement levels were always more prevalent than Vγ2 rearrangements in DEC. Since transgene rearrangement levels were roughly proportional to transgene copy number, we subsequently expressed these data as rearrangements per gene copy (Figures 3C and 3D). The data demonstrate that the transgene Vγ genes in different founder lines consistently undergo programmed rearrangement in a manner qualitatively similar to the endogenous γ genes. Since the transgenes could not encode a γ protein and therefore should not confer a selective advantage or disadvantage to the cells that rearranged them, these results provide direct evidence that Vγ gene rearrangement is differentially targeted in the progenitors of DEC versus adult DN thymocytes.

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The adult thymocyte preparations analyzed in the preceding experiments are a mixture of precursor cells and αβ and γδ lineage cells at various stages of maturation. To assess whether the patterns of rearrangement observed in these preparations also hold in the case of mature T cell populations, γδ and αβ T cell populations were examined. These populations were purified from two γSW and two γB lines by cell sorting from a mixture of splenic and lymph node cells (Table 2). The analysis showed that there was an ~5-fold predominance of transgene Vγ3 rearrangements over transgene Vγ2 rearrangements in both αβ and γδ T cells of the γSW lines. In contrast, the levels of transgene Vγ2 rearrangements were higher than transgene Vγ3 rearrangements in the two γB lines in both peripheral populations. Although the differences were small in the case of the γB lines, they were similar to the differences observed in DN thymocytes for these particular lines, which exhibited less bias than most other lines we examined (see Figure 3C). Thus, compared to the γB lines, the γSW transgene exhibited a reversed bias in the pattern Vγ rearrangements in both αβ and γδ T cells from adult peripheral lymphoid organs.

Taken together, these results demonstrate that when the Vγ3 gene was preceded by Vγ2 upstream sequences, it rearranged at Vγ2 levels in the adult thymus, and when the Vγ2 gene was preceded by Vγ3 upstream sequences, it rearranged at Vγ3 levels. Hence, it can be concluded that the sequences upstream of Vγ2 and Vγ3 provide specificity for the pattern of Vγ gene rearrangement at the adult stage of T cell development in both the γδ and αβ T cell lineages.

Vγ4 Rearrangements Do Not Increase in the γSW Lines in Adult Thymocytes

The Vγ4 gene, like the Vγ3 gene, rearranges relatively frequently in the fetal thymus and infrequently in the adult thymus. Since the Vγ4 gene is a neighbor of the Vγ3 gene, it was possible that the substitution of the Vγ2 upstream sequences for those of the Vγ3 gene in the γSW transgene might affect the rearrangement pattern of the nearby Vγ4 gene. However, we observed very little, if any, difference in the pattern of rearrangement of the Vγ4 gene in adult DN thymocytes of γSW lines when compared to control γB lines (average of 0.016 versus 0.010 rearrangements per gene copy, data not shown). In contrast, there was an average of 5.7-fold more Vγ3 rearrangements with the γSW lines compared to the γB lines. Therefore, the mechanism that enhances Vγ3 rearrangements in the adult thymus in the γSW transgene operates locally on the Vγ3 gene and does not significantly affect the nearby Vγ4 gene.

Upstream Sequences Do Not Affect the Rearrangement Pattern in DEC5 or Fetal Thymocytes

To determine whether switching the upstream regions also reversed the Vγ2 and Vγ3 rearrangement patterns in the DECs, DNA isolated from DECs was analyzed from γSW lines, and the results were compared to those from the γA and γB lines (Figure 3F). The results showed that there was not a significant difference in the levels...
of Vg2 and Vg3 rearrangements in the DECs among the γA, γB, and γSW lines. Thus, even when the Vg2 upstream sequences preceded the Vg3 gene, Vg3 still rearranged at relatively high levels, comparable to when it was preceded by its own upstream sequence. Furthermore, the Vg2 gene flanked by the Vg3 upstream region was still rearranged relatively infrequently in DECs. A similar pattern was observed in early fetal thymocytes, where a strong bias for Vg3 rearrangements was observed in the γSW transgenic lines, similar to the bias observed in the γA or γB transgenic lines (Table 1; Figure 4). These results suggest that the upstream regions do not play a central role in determining either the enhanced level of Vg3 rearrangements nor the relatively repressed level of Vg2 rearrangements observed in early fetal thymocytes and DECs.

Discussion

Programmed Rearrangement Occurs at TCRγ Locus

Our results show that mutant recombination substrates, designed so as not to encode proteins that could alter the selection fate of cells in which they rearrange, undergo programmed Vγ gene rearrangement similar to the endogenous γ genes. These data are the most direct evidence to date that targeted rearrangement is a primary determinant of the developmental pattern of Vγ gene usage. The targeting of Vγ gene rearrangements is likely to be coordinated with divergent developmental programs, such as expression of different homing receptors and cytokines. Evidence suggests that Vδ and Vγ gene rearrangements are also developmentally programmed (Chien et al., 1997; Marshall et al., 1996; ten Boekel et al., 1997; Corcoran et al., 1998).

Vγ Gene Usage Late in Development Is Controlled by Upstream Sequences

Significantly, we have been able to localize the elements that regulate Vγ gene recombination in the adult thymus to sequences 1–1.5 kb upstream of Vg2, Vg3, or both. In the case of the γSW construct, in which the upstream regions were swapped, we observed a pattern of Vg3 versus Vg2 usage in adult thymocytes that was reversed from the normal pattern, as exemplified by the γB construct. Thus, as was previously shown for enhancer elements, elements in or near the promoters of V genes can differentially regulate the timing of receptor gene rearrangement. The specificity for rearrangement in the adult thymus may lie in the sequences upstream of Vg2, Vg3, or both. It is possible that the sequences upstream of Vg2 actively promote accessibility and rearrangement of Vg2 in the adult thymus, which could confer a strong competitive advantage to Vg2 over Vg3 rearrangements in the normal gene and to Vg3 rearrangements in the γSW transgene. Alternatively, the Vg3 upstream sequence may inhibit rearrangement locally in adult thymocytes, conferring a competitive advantage to the Vg2 gene in the normal locus and to the Vg3 gene in the γSW transgene. An element(s) located between 223 bp and 897 bp upstream of the Vg3 transcriptional start site, which is contained in the swapped region, is able to repress transcription of linked promoters in transient but not stable transfections into the human γδ cell line PEER (Clausell and Tucker, 1994). While it is possible that such an element could also inhibit rearrangement, its significance is clouded by its lack of activity on chromatin substrates. Finally, it is possible that both the Vg2 and Vg3 upstream regions have roles in differentially targeting the two Vγ genes for rearrangement. The upstream regions of Vγ genes may influence rearrangement by any of several mechanisms. One possibility is that transcriptional activation of the genes by the promoters influences their ability to serve as recombination substrates. A second possibility is that transcriptional activation per se is not necessary to make the genes accessible to the recombinase but rather that the upstream elements promote or repress recombination by altering the local chromatin accessibility of the genes. While it has been demonstrated that levels of germline transcripts correlate well with the rearrangement levels of each gene during development (Goldman et al., 1993), it is possible that transcription is merely a consequence of accessible chromatin and is not required to promote recombination in this system. Studies in other systems have shown that recombination can sometimes occur in the absence of detectable germline transcripts (Kallenbach et al., 1993; Alvarez et al., 1995). Further studies will be necessary to understand the relationship between transcription and V(D)J recombination.

Does Cellular Selection Play a Role in Reinforcing the Programmed Usage of Vγ Genes?

The extent to which selection also shapes the repertoire of γδ subsets is unclear. The possibility of selection

### Table 2. Rearrangements of Vγ2 and Vγ3 Genes in γδ and γµ T Cells

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<td>γSW MEAN</td>
<td>0.050</td>
<td>0.229</td>
<td></td>
<td>0.22</td>
<td>0.035</td>
<td>0.163</td>
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*The V2 and V3 columns refer to the rearrangement/gene copy from PCR data.

The average means for the rearrangement/gene copy and V2/V3 ratios for the lines derived from each construct are noted.
acting on developing DECs was suggested by a recent study of DECs in mice in which the V\(\gamma\)3 gene was disrupted (Mallick-Wood et al., 1998). These DECs expressed V\(\gamma\) genes not found among normal DECs, yet the cells had apparently been selected for a specificity shared with the normal V\(\gamma\)3-containing DEC receptor. Another indication that selection may occur arises from a comparison of transgene V\(\gamma\)3 rearrangements in DECs versus fetal thymocytes. Although the V\(\gamma\)3 transgene in the \(\gammaB\) and \(\gammaA\) constructs rearranged relatively frequently in DECs, the average level of rearrangement was still only one-fifth the level observed for the endogenous V\(\gamma\)3 gene. This consistent discrepancy could reflect generally lower accessibility of the transgenic substrates versus the endogenous genes to the recombination machinery. Another possibility, however, is that DECs are selected for the expression of a functional TCR, increasing the representation of endogenous V\(\gamma\)3 rearrangements in these cells over the level of the unselected transgene V\(\gamma\)3 rearrangements. Implicit in this prediction is that some fetal thymocytes contain transgene rearrangements but not endogenous rearrangements, and these are the cells that are selectively depleted in the DECs. Our results with E14 fetal thymocytes were consistent with this explanation of the data. Presumably, many early fetal thymocytes have not yet undergone selection. In these cells, the average level of endogenous V\(\gamma\)3 rearrangements was nearly the same as the level of transgene rearrangements, compared to 5-fold more endogenous V\(\gamma\)3 rearrangements observed in DECs (Table 1). Although these data do not justify a definitive conclusion, they are consistent with the possibility that selection enhances the representation of endogenous V\(\gamma\)3 rearrangements in the DEC cell population. Cellular selection and programmed rearrangement can be seen as reinforcing the same outcome: to equip DECs with a T cell receptor of a defined specificity.

The levels of transgene V\(\gamma\)2 rearrangements in the adult thymus, although high, were still about 3-fold lower than the levels of endogenous V\(\gamma\)2 rearrangements. Additionally, transgene V\(\gamma\)3 rearrangements in these cells, although low, were nearly 4-fold higher than the levels of endogenous V\(\gamma\)3 rearrangements. As a result, the average ratio of V\(\gamma\)2 to V\(\gamma\)3 rearrangements (V\(\gamma\)2/V\(\gamma\)3) in the adult thymus was significantly lower for the transgene than for the endogenous gene (5.55 versus 48) (Figure 3C). It is unlikely that the higher level of endogenous V\(\gamma\)2 rearrangements in the adult thymus reflects selection for expression of V\(\gamma\)2, because the majority of V\(\gamma\)2 rearrangements are nonproductive in \(\alpha\beta\)-lineage T cells, and the adult thymus is composed primarily of \(\alpha\beta\)-lineage T cells (Kang et al., 1995). Transgene V\(\gamma\)2 rearrangements were also less frequent than endogenous rearrangements in fetal thymocytes and DECs. It is possible that the transgene lacks some control elements or is in the wrong chromatin context for optimal V\(\gamma\)2 rearrangement, although we emphasize that the transgene did rearrange quite well in the adult thymus. The somewhat elevated levels of transgene V\(\gamma\)3 rearrangements in adult thymocytes could result from tandem multimerization of the transgene, which in other systems is known to override gene repression (Dillon and Grosveld, 1991).

V\(\gamma\) Usage Early in Development Is Not Controlled by Upstream Sequences

Although the regions upstream of V\(\gamma\)2 and/or V\(\gamma\)3 dramatically influenced rearrangement of the corresponding genes in the adult thymus, they had no effect on the pattern of rearrangement in early fetal thymocytes or DECs. Thus, the early fetal pattern of rearrangement is probably imposed primarily by a mechanism that is independent of the upstream sequences. One possibility is that the rearrangement of V\(\gamma\) genes in early fetal thymocytes is influenced by the proximity of the gene to the downstream enhancer (3'E\(\gamma\)1) (Kappes et al., 1991; Spencer et al., 1991). Since V\(\gamma\)3 is the closest V\(\gamma\) gene to 3'E\(\gamma\)1, it would be more accessible to the recombinase. Later in development, either V\(\gamma\)2 would be repressed, V\(\gamma\)2 would be activated, or both. A similar mechanism is thought to control the transcription of the globin genes that are closest to the LCR early in development (Martin et al., 1996).

A second possibility is that V gene-specific regulatory sequences that control the pattern of rearrangement in the fetal thymus lie not in the upstream sequences of each gene but downstream or even within the genes. Sequences within 1 kb of the V\(\gamma\)2 and V\(\gamma\)3 RSSs in the transgene are weakly hypersensitive to cleavage with DNase 1 (data not shown), suggesting that regulatory elements could lie downstream of the genes. It has also been shown that certain sequences immediately adjacent to RSSs as well as the sequences of the RSSs themselves can influence the frequency at which gene segments recombine (Ramsden and Wu, 1991; Gerstein and Lieber, 1993; Ezekiel et al., 1995). Finally, we have recently identified a second enhancer-like element in the \(\gamma\) locus, that is present in the transgenes and that stimulates both recombination and transcription (J. E. B., J. Kang, T. Chen, D. C., and D. H. R., submitted). We are currently investigating the roles of the novel element, 3'E\(\gamma\)1 and sequences linked to each V gene in creating the pattern of V gene recombination observed in the early fetal thymus.

Experimental Procedures

Transgenic Mice

The transgenes used were assembled from BALB/c DNA fragments from phage clones (Garman et al., 1986) (P. Doherty and D. H. R., unpublished data) and cosmid clones (Vernooy et al., 1993) kindly provided by Drs. Kai Wang and Lee Hood. The assembled transgenes are all contiguous genomic sequences with no missing intervals. \(\gammaA\) is a 50 kb construct extending from the EcoRI site 4 kb upstream of V\(\gamma\)2 to a Sau3AI site 15 kb downstream of the third C\(\gamma\)1 exon. The Clal site in the V\(\gamma\)2 coding region was destroyed by digesting with Clal and filling in the recessed ends with Klenow, which created a novel NruI site. The mutations in the V\(\gamma\)4 and V\(\gamma\)3 coding regions have been previously described (Asarnow et al., 1993). These frameshift mutations resulted in termination codons prior to the RSS. All other sequences in the construct were identical to the endogenous locus. \(\gammaB\) lacks 11 kb at the 3' end compared to \(\gammaA\), ending at the SalI site; otherwise, \(\gammaB\) and \(\gammaA\) are identical. \(\gammaSW\) is identical to \(\gammaB\) except the 1.5 kb SpeI/HindIII fragment of the V\(\gamma\)2 upstream sequence was reciprocally exchanged with the 1.1 kb HindIII/EcoRV fragment containing V\(\gamma\)3 upstream sequences, destroying all four sites.

The transgene constructs, free of vector DNA, were injected into fertilized (C57BL/6XCBAJ) F2 eggs. Transgenic founders, identified by blotting tail DNA, were either analyzed directly or were backcrossed repeatedly to CBA/J mice (purchased from the National Immunity 166
Programmed Rearrangement of TCR Vγ Genes

Cancer Institute) to generate transgenic lines. Transgene copy number was determined by quantitative blotting of tail DNA. In the cases where examined, transgene copy number in thymus DNA was similar to that in tail DNA.

Cell Preparations for DNA Analysis

CD4+CD8+ thymocytes were prepared by complement lysis of whole thymocytes with anti-CD4 (RL172) and anti-CD8 (3.168.8) antibodies and a mixture of guinea pig complement (GIBCO Laboratories) and rabbit sera, followed by isolation of live cells on a Ficoll gradient. Gestational ages of fetuses were determined as the number of days after a vaginal plug was observed (Day 0). The whole fetal thymus, including the capsule, was used to isolate DNA. To purify γδ and γ T cells, spleen cells were depleted of red blood cells and combined with lymph node cells and then passed over nylon wool to deplete B cells and macrophages. The resulting cells were sorted on a Coulter Epics Elite flow cytometer using anti-γ (GL3-FITC) and anti-δ (H57-biotin) antibodies. DECs were prepared from murine skin as described (Sullivan et al., 1985).

DNA Preparation

Genomic DNA from defined numbers of cells was prepared as described (Asabuel et al., 1991) except the digestion buffer contained 10 mM Tris (pH 7.5), 0.15 M NaCl, 25 mM EDTA, 400 μg/ml proteinase K, and 0.1% SDS; no additional salt was added during the precipitation step, and 2.5 μg of lambda DNA (New England BioLabs) was added as carrier.

PCR Primers

The L2, L3, L4, 3′-tubulin, 5′-tubulin, and J1 primers have been previously described (Asarnow et al., 1989; Goldman et al., 1993). Other PCR primer sequences are as follows: GACCAAAACAGCGG TGACACATAAG (PSVγ3), AGAAGGTGAACCATACACTGTAGCC (PSVγ2), and CTTAGTCTTCTGCAATTACCTGGTG (PS γ1).

Semi-quantitative PCR of DNA

Serial 3-fold dilutions of DNA were prepared in the presence of 50 μg/ml bacteriophage lambda DNA (New England Biolabs). The PCR reaction was performed in a final volume of 50 μl containing 25 pmol of each primer, 200 μM each of dNTP, 1.5 mM MgCl2, 1× PCR buffer (Promega), 1.25 U of Taq DNA polymerase (Promega), 1 μCi of α-32PdCTP, and 1 μg/ml lambda DNA. The samples were incubated at 94°C for 3 min, followed by amplification for 28 cycles at 94°C (1 min), 55°C (1 min), and 72°C (1 min + 2 sec each additional cycle), ending with a 10 min incubation at 72°C. 10 μl of each sample was digested with the appropriate transgene-specific restriction enzyme and run on either a 5% or 10% polyacrylamide gel. The bands were visualized by autoradiography, and their intensities were measured on a Phosphorimager.

Quantitation of the rearrangement levels in the initial nucleic acid sample was accomplished based on comparison to the standard cell lines WRD.34 and DN2.3. WRD.34 is a Vγ3+ DEC line derived from BALB/c mice, kindly provided by Michael Kuhns. The DN2.3 cell line was described previously (Marusic-Galesic et al., 1989). A standard curve was constructed using the dilutions from the standard cell line, and the levels of Vγ gene rearrangements in the test samples were determined by comparing the band intensities to the standard curve, after accounting for the different sizes of the amplified fragments. The dilutions were also amplified with β-tubulin-specific primers, and the starting DNA levels were normalized by comparing the levels of β-tubulin DNA in the test and standard samples. The calculations were based on the WRD.34 cell line containing two Vγ3 rearranged genes and two tubulin genes and the DN2.3 hybridoma containing two Vγ2 rearranged genes and four tubulin genes. The values obtained for the initial dilutions were averaged, except in instances when it was clear that a particular dilution was aberrant. The values presented in the results section represent one analysis or the average from two or three independent assays of the same DNA preparations. When multiple assays were performed, the range of the data was typically within ±25% of the average. Only 10% or so of the epidermal cell preparations were Vγ3+ DEC. However, it proved difficult to accurately estimate this fraction because the epidermal cell preparations often formed large aggregates, complicating flow cytometry. Each Vγ3+ DEC must have one or two endogenous Vγ3 rearrangements. In a few cases, we determined Vγ3 rearrangement levels in sorted Vγ3+ DECs in comparison to cell lines with known numbers of Vγ3 rearrangements. The results showed that an average of approximately one Vγ3 gene was rearranged per DEC cell, corresponding to 0.5 rearrangements per gene copy. Therefore, when analyzing each unsorted epidermal cell preparation, we normalized all of the values (transgenic Vγ3 rearrangements and endogenous and transgenic Vγ2 rearrangements) based on a level of 0.5 endogenous Vγ3 rearrangements per gene copy.

Southern Blot Analysis

Southern blot analysis was performed essentially as described (Ausubel et al., 1991). The membranes were washed at 65°C with 0.5× SSC and 1% SDS.

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References


