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Developmentally Programmed Rearrangement of T Cell Receptor Vγ Genes Is Controlled by Sequences Immediately Upstream of the Vγ Genes

Jeanne E. Baker, Dragana Cado, and David H. Raulet*
Department of Molecular and Cell Biology
Cancer Research Laboratory
University of California at Berkeley
Berkeley, California 94720

Summary

Distinct subsets of γδ T cells expressing different Vγ and Vδ chains arise in ordered waves during thymic development. In the murine Jγ1–Cγ1 cluster, the Vγ3 gene segment is utilized earliest in fetal thymic development, in progenitors of dendritic epidermal T cells (DECs). The Vγ2 gene segment predominates in the late fetal stages and beyond, in cells destined for the secondary lymphoid organs. Using transgenic TCRγ recombination substrates, we demonstrate that this restricted Vγ gene usage is determined by developmentally targeted gene rearrangement. We show that sequences immediately upstream of the Vγ2 and Vγ3 genes direct the rearrangement pattern in adult thymocytes. Thus, the choice of Vγ gene 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 recombination 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 TCRγ, -β, and -δ precedes TCR α rearrangement, while in B cells, IgH rearrangement occurs at an earlier developmental stage than Igκ or -λ. In the case of IgH and TCRβ genes, D to J rearrangement precedes V to DJ rearrangement.

A special case of developmentally regulated V(D)J recombination 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-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 Vγ genes is even more strikingly regulated. A developmental switch in the late fetal stage results in the predominant rearrangement of a distinct set of Vγ 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 Cγ1 cluster of the TCRγ locus contains four closely linked but distantly related variable region genes in the following order: Vγ5-Vγ2-Vγ4-Vγ3, with Vγ3 being the most Jγ1-proximal. Vγ3 and Vγ4 rearrangements are most prevalent in the early fetal thymus and are very rare in the adult thymus, whereas Vγ2 and Vγ5 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. $V\gamma3^+$ 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 $V\gamma4^+$ cells are also only present in the fetal thymus (Ito et al., 1989). $V\gamma2^+$ γδ cells, and probably $V\gamma5^+$ 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 Vγ genes generally exhibit distinct functional characteristics. The $V\gamma3^+$ 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 $V\gamma3^+$ 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 $V\gamma4^+$ 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 $V\gamma3^+$ and $V\gamma4^+$ cells and the absence of V(D)J junctional diversity in both γ and δ chains, each population expresses only a single receptor specificity (Asarnow 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 $V\gamma3^+$ and $V\gamma4^+$ subsets, $V\gamma2^+$ 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 Vγ2 subset, it has been proposed that these cells recognize foreign antigens (Raulet, 1989). The different properties and localization of the Vγ-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

*To whom correspondence should be addressed (e-mail: raulet@uclink4.berkeley.edu).

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 $V\gamma3^+$ or $V\gamma2^+$ 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 (Raulet et al., 1991; Heyborne et al., 1993). Second, at the Cγ1 cluster, there is a strong correlation between the timing of germline 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 germline transcription is somehow related to the accessibility of gene segments to the recombinase (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 recombinase. It has been proposed that the lineage-specificity and stage-specificity of V(D)J recombination at each antigen receptor gene in B and T cells is regulated at the level of accessibility (Sleckman et al., 1996; Stanhope-Baker et al., 1996). Substantial evidence demonstrates that *cis*-acting DNA elements that regulate transcription, particularly enhancers, also play a large role in regulating V(D)J recombination. Experiments using both transgenic recombination substrates and targeted deletions demonstrate that enhancers are almost always required for normal levels of recombination in vivo (Sleckman et al., 1996). In addition, various enhancers are able to control the stage specificity as well as the cell type specificity of rearrangement (Capone et al., 1993; Lauzurica and Krangel, 1994). However, none of the studies done to date address the mechanisms underlying a developmental switch in the choice of several closely linked V region genes for recombination.

One possible explanation of the developmental regulation of Vγ gene usage is that V-proximal regulatory elements differentially regulate Vγ gene accessibility at different developmental stages or in distinct progenitor populations. The promoter is the best characterized local *cis*-acting element, containing sequences that are necessary for recruitment of the transcriptional machinery as well as binding sites for various regulatory proteins. The sequences upstream of Vγ2, Vγ3, and Vγ4 are quite divergent (P. Doherty and D. H. R., unpublished data), suggesting that each promoter could be regulated by distinct *trans*-acting factors. The Vγ3 promoter has been identified using transient transfection assays and contains potential negative regulatory elements (Clausell and Tucker, 1994), while the Vγ2 promoter has yet to be precisely defined.

We employed transgenic recombination substrates to directly address whether Vγ gene rearrangement is programmed and is regulated by *cis*-acting elements. The transgenes can be considered "reporter" constructs since they were rendered incapable of encoding functional γ chains and should therefore not alter the selection status of the T cells in which they recombine. We show that the transgenic substrates faithfully recapitulate the developmental pattern typical of endogenous Vγ genes, directly demonstrating that the pattern is due to targeted V gene recombination. More importantly, we also demonstrate that the pattern of recombination in the adult thymus is determined by sequences in or near the promoters of the Vγ2 and Vγ3 genes.

Results

Experimental Approach

Two recombination substrates were constructed that encompass most of the Cγ1 cluster (Figure 1A). γA is 50 kb in length and extends from 4 kb upstream of Vγ2 to 15 kb downstream of the third Cγ1 exon. γB is identical to γA, except that it lacks 11 kb on the 3′ end. 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 Jγ1 (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,

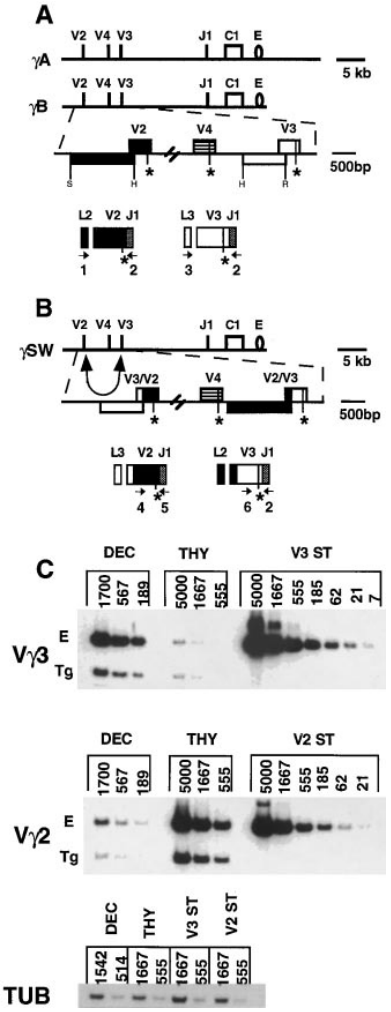

Figure 1. Rearrangement of TCRγ Gene Transgenes

(A and B) Schematic structures of (A) γA and γB and (B) γSW are depicted. The 3′ enhancer (3′$E_{Cγ1}$) 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 Vγ2 sequences are indicated in black and Vγ3 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 Vγ3 and Vγ2 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.

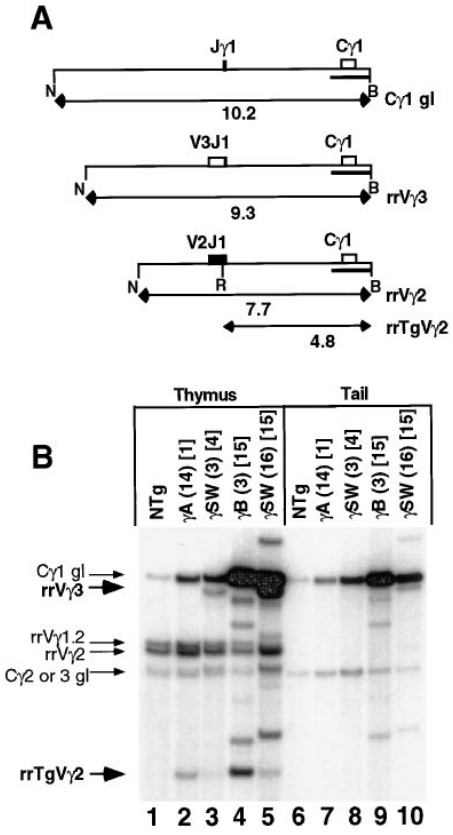

Figure 2. Southern Hybridization Analysis of Vγ Rearrangements in Unfractionated Transgenic Thymocytes Versus Tail DNA

(A) Map of pertinent regions from the Cγ1 locus. The DNA was digested with NcoI (N), NruI (R), and BamHI (B). The probe was generated from the first exon of the Cγ1 constant region and is indicated by a thick line under the Cγ1 exon. This probe hybridizes to the Cγ2 and Cγ3 constant regions as well as to Cγ1. Distances in kb between sites are indicated.

(B) Southern analysis. The identities of the bands are noted to the left. The transgenic lines are indicated, with the transgene copy number in brackets. Since Vγ3 rearrangements are undetectable in adult thymocytes from nontransgenic mice, it is assumed that the rearranged Vγ3 band corresponds to the transgene. The identity of the additional bands in the γB(3) and γSW(16) lanes is unknown, but they probably reflect transgene integration sites since they are identical in both thymus and tail. Abbreviations: Ntg, nontransgenic; Tg, transgene; gl, germline; rr, rearranged.

we compared rearrangement levels in adult $CD4^-CD8^-$ (double negative or DN) thymocytes, which contain essentially all of the thymic γδ cells, and in adult epidermal epithelial cell preparations that contain the $Vγ3^+$ DECs. The latter population arises from the fetal thymic $Vγ3^+$ progenitors.

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

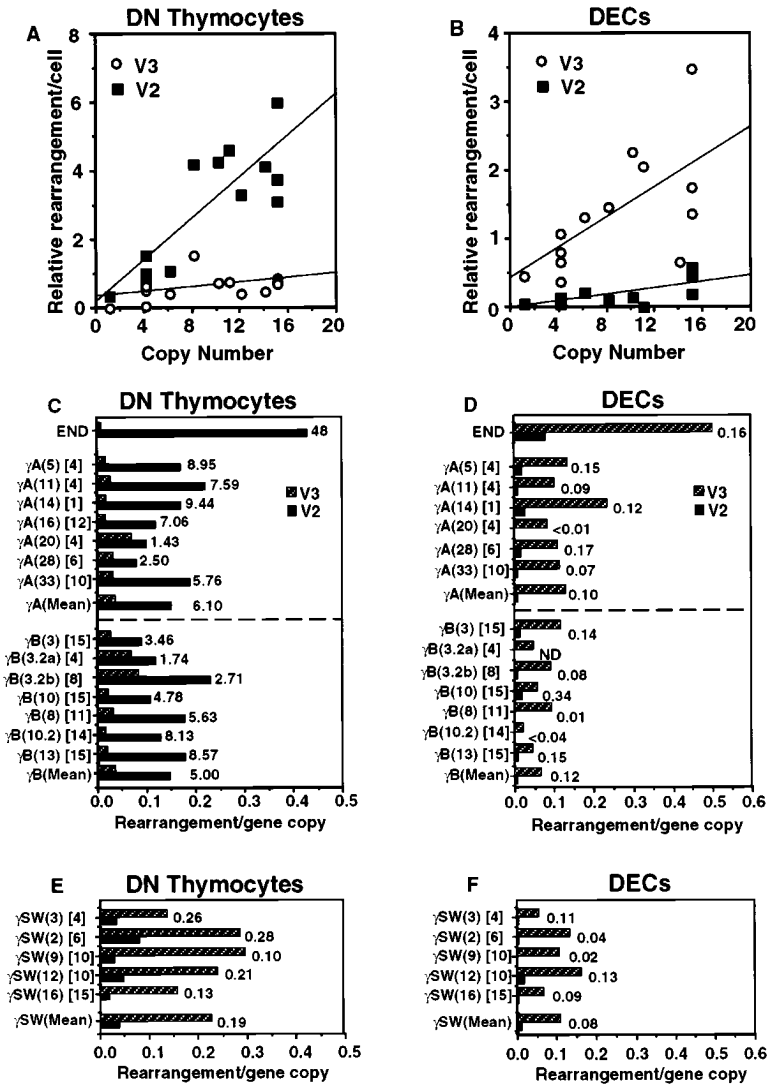

Figure 3. Quantitation of Transgene Rearrangements in Thymocytes and DECs

(A and B) Transgene rearrangements in thymocytes and DECs are roughly proportional to transgene copy number. Each point represents an individual γA or γB transgenic line. A unit of one on the y-axis corresponds to the level of rearrangement per cell of each endogenous Vγ2 gene in adult thymocytes (A) and each endogenous Vγ3 gene in DECs (B).

(C and D) Transgene rearrangement levels per transgene copy in adult DN thymocytes and DECs for each γA and γB transgenic line.

(E and F) Transgene rearrangement levels per transgene copy in adult DN thymocytes and DECs for each γSW transgenic line. Transgene copy numbers are in brackets. END refers to the endogenous Vγ genes, where the number of gene copies is two. The ratios of Vγ2/Vγ3 rearrangements per gene copy are denoted to the right of each set of bars. Vγ2 rearrangements were not calculated for the γB(3.2a) line in the DEC analysis.

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 Vγ2 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 αβ lineage cells, these cells usually harbor rearrangements at the Jγ1-Cγ1 locus, most of which are Vγ2 rearrangements. Therefore, total thymocytes and thymic DN cells exhibit a similar pattern of TCRγ rearrangements (Garman et al., 1986). Lane 2 in Figure 2 corresponds to the γA(14) line harboring one transgene copy (in the figures, transgene copy number is indicated by brackets). Transgene Vγ2 rearrangements were observed in total thymocytes, whereas Vγ3 rearrangements were undetectable (Figure 2, lane 2). Similarly, in the 15-copy γB(3) line, a strong band indicating transgene Vγ2 rearrangements was evident, but no transgene Vγ3 rearrangements were detectable (Figure 2, lane 4). Hence, the added 3′ sequences in the γA construct compared to the γB 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 Vγ2 rearrangements over Vγ3 rearrangements in the adult thymus is even greater than suggested by the PCR results (compare to Figure 3C below). The stronger signal for Vγ2 rearrangements evident in the γB(3) transgenic mice versus the γA(14) transgenic mice presumably reflects the higher number of transgene copies available to undergo rearrangement in the γB(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 γA and γB 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 Vγ2 gene in adult thymocytes (Figure 3A) or one endogenous Vγ3 gene in DECs (Figure 3B). The graphs revealed

Table 1. Rearrangements of Vγ2 and Vγ3 Genes in Fetal Thymocytes

		DAY14 FETAL THYMUS			DAY15 FETAL THYMUS		
LINE	COPY #	V2[a]	V3[a]	V2/V3[b]	V2[a]	V3[a]	V2/V3[b]
END	2	0.00120	0.00330	0.364	0.0247	0.0930	0.266
γA(14)	1	<0.00001	0.00400	<0.003	ND	ND	ND
γA(20)	4	0.00021	0.00220	0.095	ND	ND	ND
γA(33)	10	0.00002	0.00125	0.016	ND	ND	ND
γA MEAN		0.00008	0.00248	0.037			
γB(3)	15	0.00008	0.00590	0.014	0.0023	0.0300	0.077
γB(3.2b)	8	0.00002	0.00060	0.033	0.0009	0.0080	0.113
γB MEAN		0.00005	0.00325	0.024	0.0016	0.0190	0.095
γSW(3)	4	0.00004	0.00210	0.019	0.0018	0.1070	0.017
γSW(12)	10	0.00001	0.00090	0.011	0.0018	0.0290	0.062
γSW MEAN		0.00003	0.00150	0.015	0.0018	0.0680	0.040

[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 DECs. 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 DECs 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 DECs. 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 DECs 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 DECs, 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 Vγ2 and Vγ3 genes were reciprocally exchanged. Previous evidence suggested that the Vγ3 promoter required sequences in the intron between the leader and coding exons to exhibit demonstrable activity in transient transfection assays (Clausell and Tucker, 1994). This observation suggested that some regulatory sequences might be present within this intron. Therefore, the region that was swapped included 900 bp of Vγ3 upstream sequences and in addition the leader exon of Vγ3, the intron, and a few base pairs of the Vγ3 coding exon (Figure 1B). To maintain a reciprocal gene structure, the Vγ2 upstream region that was exchanged also included the corresponding regions of the Vγ2 gene and extended 1.2 kb upstream of the Vγ2 leader exon (Figure

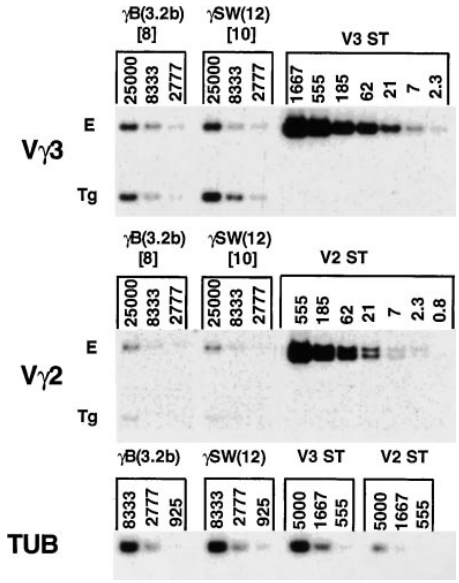

Figure 4. Semiquantitative PCR Analysis of Day 14 Fetal Thymocyte DNA from One γB Line and One γSW Line

The transgene copy number of each line is indicated in brackets. The primers used were PSVγ3/J1 (Vγ3), PSVγ2/PSJγ1 (Vγ2), and 5′ and 3′ β tubulin (TUB). See Figure 1 legend for other details.

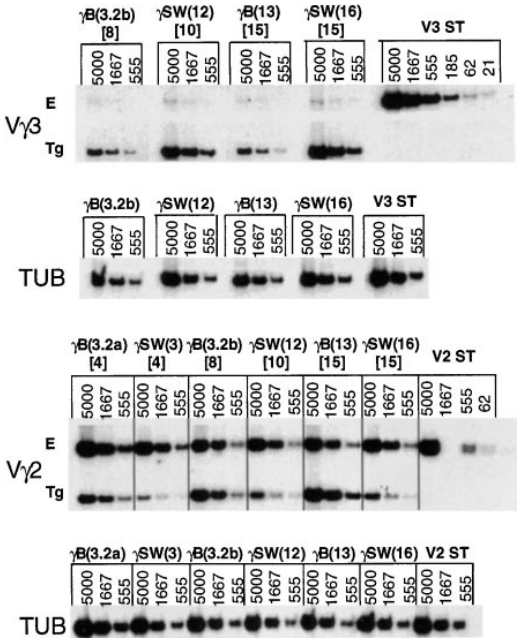

Figure 5. Semiquantitative PCR Analysis of Adult DN Thymocyte DNA from Several γB and γSW Lines

The results are organized so that lines of similar transgene copy number (indicated in brackets) are adjacent to each other. The primers used were PSVγ3/J1 (Vγ3), PSVγ2/PSJγ1 (Vγ2), and 5′ and 3′ β tubulin (TUB). See Figure 1 legend for other details.

1B). Five transgenic founders were obtained from the γSW construct, and each was bred to generate a transgenic line.

Figure 5 compares semiquantitative PCR analysis of Vγ2 and Vγ3 rearrangements in adult DN thymocytes of several γSW lines versus several γB lines. In each case, a γSW line is compared directly to a γB line of similar transgene copy number. Results for all of the lines are summarized in Figure 3E. The data reveal that the γSW transgenes exhibit an opposite pattern of rearrangement as the γB transgenes in the adult DN thymocytes. Vγ3 rearrangements from the γSW construct were elevated to the level of Vγ2 rearrangements from the γB construct. Conversely, Vγ2 rearrangements from the γSW construct were depressed to near the level of Vγ3 rearrangements in the γB construct. As a consequence, the average Vγ2/Vγ3 rearrangement ratios in the γB and γSW lines were nearly precise reciprocals (5.00 versus 0.19) (Figures 3C and 3E).

These results were corroborated by Southern blotting of DNA from unfractionated adult thymocytes, in which we compared transgenic lines of similar transgene copy numbers representing the control and γSW constructs (Figure 2B). Lane 2 reveals detectable transgene Vγ2 rearrangements in the single copy γA(14) line but no detectable transgene Vγ3 rearrangements, whereas the four-copy γSW(3) line in lane 3 exhibits the opposite pattern. The comparison of the 15-copy γB(3) line in lane 4 with the 15-copy γSW(16) line in lane 5 also reveals reciprocal patterns of Vγ2 and Vγ3 rearrangements, though the bands are more intense, as expected due to a higher number of available substrates for recombination.

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 DECs 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

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

		αβ			γδ		
LINE	COPY #	V2[a]	V3[a]	V2/V3[b]	V2[a]	V3[a]	V2/V3[b]
END	2	0.470	0.022	21.36	0.380	0.015	25.33
γB(3)	15	0.210	0.088	2.39	0.140	0.050	2.80
γB(3.2b)	8	0.120	0.073	1.64	0.100	0.047	2.13
γB MEAN		0.170	0.081	2.02	0.120	0.049	2.47
γSW(3)	4	0.040	0.192	0.21	0.030	0.143	0.21
γSW(16)	15	0.060	0.265	0.23	0.040	0.183	0.22
γSW MEAN		0.050	0.229	0.22	0.035	0.163	0.22

[a] The V2 and V3 columns refer to the rearrangment/gene copy from PCR data.
[b] The V2/V3 columns represent the ratio of V2 to V3 rearrangement/gene copy.
The average means for the rearrangement/gene copy and V2/V3 ratios for the lines derived from each construct are noted.

of Vγ2 and Vγ3 rearrangements in the DECs among the γA, γB, and γSW lines. Thus, even when the Vγ2 upstream sequences preceded the Vγ3 gene, Vγ3 still rearranged at relatively high levels, comparable to when it was preceded by its own upstream sequence. Furthermore, the Vγ2 gene flanked by the Vγ3 upstream region was still rearranged relatively infrequently in DECs. A similar pattern was observed in early fetal thymocytes, where a strong bias for Vγ3 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 Vγ3 rearrangements nor the relatively repressed level of Vγ2 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 V_H gene rearrangements are also developmentally programmed (Chien et al., 1987; 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 Vγ2, Vγ3, or both. In the case of the γSW construct, in which the upstream regions were swapped, we observed a pattern of Vγ3 versus Vγ2 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 Vγ2, Vγ3, or both. It is possible that the sequences upstream of Vγ2 actively promote accessibility and rearrangement of Vγ2 in the adult thymus, which could confer a strong competitive advantage to Vγ2 over Vγ3 rearrangements in the normal gene and to Vγ3 rearrangements in the γSW transgene. Alternatively, the Vγ3 upstream sequence may inhibit rearrangement locally in adult thymocytes, conferring a competitive advantage to the Vγ2 gene in the normal locus and to the Vγ3 gene in the γSW transgene. An element(s) located between 223 bp and 897 bp upstream of the Vγ3 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 Vγ2 and Vγ3 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

acting on developing DECs was suggested by a recent study of DECs in mice in which the Vγ3 gene was disrupted (Mallick-Wood et al., 1998). These DECs expressed Vγ genes not found among normal DECs, yet the cells had apparently been selected for a specificity shared with the normal Vγ3-containing DEC receptor. Another indication that selection may occur arises from a comparison of transgene Vγ3 rearrangements in DECs versus fetal thymocytes. Although the Vγ3 transgene in the γB and γA constructs rearranged relatively frequently in DECs, the average level of rearrangement was still only one-fifth the level observed for the endogenous Vγ3 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 Vγ3 rearrangements in these cells over the level of the unselected transgene Vγ3 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 Vγ3 rearrangements was nearly the same as the level of transgene rearrangements, compared to 5-fold more endogenous Vγ3 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 Vγ3 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 Vγ2 rearrangements in the adult thymus, although high, were still about 3-fold lower than the levels of endogenous Vγ2 rearrangements. Additionally, transgene Vγ3 rearrangements in these cells, although low, were nearly 4-fold higher than the levels of endogenous Vγ3 rearrangements. As a result, the average ratio of Vγ2 to Vγ3 rearrangements (Vγ2/Vγ3) 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 Vγ2 rearrangements in the adult thymus reflects selection for expression of Vγ2, because the majority of Vγ2 rearrangements are nonproductive in αβ-lineage T cells, and the adult thymus is composed primarily of αβ-lineage T cells (Kang et al., 1995). Transgene Vγ2 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 Vγ2 rearrangement, although we emphasize that the transgene did rearrange quite well in the adult thymus. The somewhat elevated levels of transgene Vγ3 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).

Vγ Usage Early in Development Is Not Controlled by Upstream Sequences

Although the regions upstream of Vγ2 and/or Vγ3 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 Vγ genes in early fetal thymocytes is influenced by the proximity of the gene to the downstream enhancer ($3'E_{C\gamma1}$) (Kappes et al., 1991; Spencer et al., 1991). Since Vγ3 is the closest Vγ gene to $3'E_{C\gamma1}$, it would be more accessible to the recombinase. Later in development, either Vγ3 would be repressed, Vγ2 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 Vγ2 and Vγ3 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 γ 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, $3'E_{C\gamma1}$ 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 (Vernooij et al., 1993) kindly provided by Drs. Kai Wang and Lee Hood. The assembled transgenes are all contiguous genomic sequences with no missing intervals. γA is a 50 kb construct extending from the EcoRI site 4 kb upstream of Vγ2 to a Sau3A site 15 kb downstream of the third Cγ1 exon. The ClaI site in the Vγ2 coding region was destroyed by digesting with ClaI and filling in the recessed ends with Klenow, which created a novel NruI site. The mutations in the Vγ4 and Vγ3 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. γB lacks 11 kb at the 3′ end compared to γA, ending at the SalI site; otherwise, γB and γA are identical. γSW is identical to γB except the 1.5 kb SpeI/HindIII fragment of the Vγ2 upstream sequence was reciprocally exchanged with the 1.1 kb HindIII/EcoRV fragment containing Vγ3 upstream sequences, destroying all four sites.

The transgene constructs, free of vector DNA, were injected into fertilized (C57BL/6XCBA/J)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

Cancer Institute) to generate transgenic lines. Transgene copy number was determined by quantitative blotting of tail DNA. In the cases 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 αβ and γδ 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-γδ (GL3-FITC) and anti-αβ (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 (Ausubel 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′β-tubulin, 5′β-tubulin, and J1 primers have been previously described (Asarnow et al., 1989; Goldman et al., 1993). Other PCR primer sequences are as follows: GACCAAACAAGACGG TGCACATAAG (PSVγ3), AGGAGTGTAACCATACACTGGTACCG (PSVγ2), and CTTAGTTCCTTCTGCAAATACCTTGTG (PSJγ1).

Semiquantitative 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 pmoles of each primer, 200 μM each of dNTP, 1.5 mM $MgCl_2$, 1× PCR buffer (Promega), 1.25 U of Taq DNA polymerase (Promega), 1 μCi of αP^{32}dCTP, and 5 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°. 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 $V\gamma3^+$ DEC line derived from BALB/c mice, kindly provided by Michael Kuhns. The DN2.3 cell line was described previously (Marusic-Galesic et al., 1988). A standard curve was constructed using the dilutions from the standard cell line, and the levels of V 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 individual 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 $V\gamma3^+$ DECs. However, it proved difficult to accurately estimate this fraction because the epidermal cell preparations often formed large aggregates, complicating flow cytometry. Each $V\gamma3^+$ DEC must have one or two endogenous Vγ3 rearrangements. In a few cases, we determined Vγ3 rearrangement levels in sorted $V\gamma3^+$ 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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