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Distinct Control of the Frequency and Allelic Exclusion of the Vβ Gene Rearrangement at the TCRβ Locus[1]

Ping Sieh[2] and Jianzhu Chen[3]

Ag receptor gene loci contain many V gene segments, each of which is recombined and expressed at a different frequency and is subject to allelic exclusion. To probe the parameters that mediate the different levels of regulation of V gene rearrangement, a Vβ gene segment together with 3.6-kb 5′ and 0.7-kb 3′ flanking sequences was inserted 6.8 kb upstream of the Dβ1 gene segment in the murine TCRβ locus. Despite its proximity to the Dβ gene segments and the Eβ enhancer, the inserted Vβ segment underwent VDJ recombination at the same frequency as the natural copy located 470 kb upstream. However, the inserted Vβ segment was no longer under allelic exclusion control as it recombined at a similar frequency in the presence of a TCRβ transgene. These results suggest that while the inserted fragment contains the necessary *cis*-regulatory elements for determining the frequency of Vβ rearrangement, additional *cis*-regulatory elements are required for mediating Vβ allelic exclusion. Interestingly, most of the inserted Vβ rearrangements were not transcribed and expressed in the presence of a TCRβ transgene, suggesting that TCRβ allelic exclusion can also be achieved by blocking the transcription of the rearranged gene segments. These findings provide strong evidence for distinct control of the frequency and allelic exclusion of Vβ gene rearrangement. ***The Journal of Immunology,* 2001, 167: 2121–2129.**

Recombination of V(D)J, by which the primary Ag receptor diversity is generated, is a highly regulated process. At the TCRβ locus, recombination occurs at the $CD4^-CD8^-CD44^-CD25^+$ stage of thymocyte development (1). In general, Dβ to Jβ rearrangement precedes Vβ to DβJβ rearrangement and occurs on both alleles, whereas Vβ to DβJβ rearrangement appears to occur on one allele at a time (2). If the initial VβDβJβ rearrangement is nonproductive, then Vβ to DβJβ rearrangement can proceed on the other allele. If the first VβDβJβ rearrangement is productive, Vβ to DβJβ joining on the second allele is inhibited. Thus, an individual T cell expresses a single TCRβ-chain, a phenomenon known as allelic exclusion. In addition, both human and mouse TCRβ loci contain multiple Vβ gene segments. Although individual Vβs are rearranged and expressed at different frequencies, a given Vβ gene segment is recombined and expressed at a constant frequency as most clearly shown in different mice of the same strain (3). This report investigates the parameters that regulate the frequency, timing, order, and allelic exclusion of Vβ gene rearrangement.

V(D)J recombination at different Ag receptor gene loci is mediated by the same recombinase machinery and conserved recombination signal sequences (RSS).[4] The various levels of regulation of V(D)J recombination are thought to be mediated through the control of accessibility of substrate gene segments (4, 5). Studies have shown that *cis*-regulatory elements that activate transcription of germline gene segments also promote their recombination accessibility (reviewed in Refs. 6 and 7). For example, deletion of the transcriptional enhancer Eβ from the endogenous TCRβ locus in mice impairs both germline transcription and rearrangement of Dβ and Jβ gene segments (8–10). Conversely, inclusion of the Eβ in recombination substrates promotes germline transcription as well as rearrangements of both Dβ to Jβ and Vβ to DβJβ in transgenic mice (11, 12). In contrast to the long-range effect of Eβ, deletion of the germline promoter PDβ1, located immediately upstream of the Dβ1 gene segment (13, 14), impairs germline transcription and rearrangement of the Dβ1 without affecting germline transcription and rearrangement of Dβ2, Jβ2, and Vβ gene segments (15, 16). Similarly, Eα is required for rearrangement of all Jα gene segments at the TCRα locus, while T early α promoter is required for the rearrangement of only proximal Jα gene segments (17, 18). Although the precise role of transcription in recombination accessibility has yet to be elucidated, these findings suggest that promoter-enhancer interaction may be a general mechanism for targeting recombination accessibility of specific gene segment.

Assuming that accessibility control applies to the Vβ gene rearrangement, at least three types of regulation could be envisioned. First, the various levels of regulation of Vβ rearrangement could be mediated by a single master *cis*-regulatory element. This possibility is not likely because it cannot account for the different frequencies of Vβ rearrangements. Second, individual Vβs could be regulated separately, for example by their own specific promoters. Although this kind of regulation can readily account for the different frequencies of Vβ rearrangements, it may not be most efficient for achieving the ordered, stage- and allele-specific Vβ rearrangement. The third possibility, a combination of the two extremes, appears to be more likely. In this scenario, rearrangement of each Vβ is regulated by an individual *cis* element, such as a promoter, as well as one or more common *cis* elements, such as enhancers, analogous to the accessibility control of the Dβ1 by both PDβ1 promoter and Eβ enhancer. The individual elements

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[4] Abbreviations used in this paper: RSS, recombination signal sequence; ES, embryonic stem; PGK, phosphoglycerate kinase; neo, neomycin; RAG, recombination-activating gene; DN, double negative.

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may determine the frequencies of Vβ rearrangements, while the common element may control the order, timing, and allelic exclusion of Vβ gene rearrangements.

Although transcriptional activation of Vβ often precedes their rearrangement and the occurrence of allelic exclusion is associated with a down-regulation of Vβ germline transcription (19, 20), studies that examine the role of Vβ promoter and Eβ enhancer in Vβ rearrangement have so far been inconclusive. In experiments using a TCRβ minilocus in transgenic mice, a Vβ was shown to recombine in the absence of germline transcription (21), although the observed rearrangement may have been promoted by a Vβ promoter present 2.5 kb upstream in the same construct. It is also possible that different *cis*-regulatory elements in the promoter may function in transcription and recombination as observed for enhancers (22, 23). Similarly, the dependence on Eβ for Vβ to DβJβ rearrangement in recombination substrates may have been complicated by integration site influences, concatenation of substrate, and changes in distance and configuration of Vβ to Dβ and Eβ in the substrate (24). In $E\beta^{-/-}$ mice, the impaired Vβ to DβJβ rearrangement could be a result of a block in accessibility of Vβ, or Dβ, or both. In the absence of Eβ, the Dβ-Jβ region, but not the Vβ region, was silent in germline transcription and resistant to endonuclease treatment (10), indicating that Eβ may not directly regulate Vβ transcription or recombination accessibility. Whether there are long-range acting *cis*-regulatory elements for regulating Vβ recombination is not known.

To probe the parameters that regulate the different levels of Vβ rearrangement, we inserted a Vβ gene segment, together with its endogenous promoter and RSS, just 6.8 kb upstream of the Dβ1 gene segment at the TCRβ locus in mice. We found that the inserted Vβ gene segment recombined at the same frequency as the natural copy but its rearrangement was no longer inhibited by the presence of a functional TCRβ transgene. These findings support a regulatory mechanism by which distinct *cis*-regulatory elements control the frequency and allelic exclusion of Vβ rearrangement.

Materials and Methods

Targeting construct and chimeric and germline mutant mice

Homologous recombination in embryonic stem (ES) cells followed by Cre/loxP-mediated deletion was used to insert a copy of the Vβ13 gene segment at the *Spe*I site 6.8 kb upstream of the Dβ1 gene segment (see Fig. 1*A*). Previously, we showed that the three DNase I-hypersensitive sites within a 3-kb region upstream of Dβ1 were required for efficient Dβ1 rearrangement (15). The insertion was ~4 kb upstream of the most 5′ DNase I-hypersensitive site and was not expected to affect Dβ1 rearrangement. The inserted 4.8-kb *Pst*I-*Sna*BI DNA fragment contains Vβ13 and 3.6-kb and 0.7-kb flanking sequences at 5′ and 3′, respectively, to ensure the inclusion of the endogenous Vβ13 promoter and RSS. The targeting vector contained, in the following order, a 5-kb *Spe*I fragment as the 5′ homologous sequences, the 4.8-kb Vβ13 fragment, a phosphoglycerate kinase (PGK) promoter-driven neomycin (neo) resistance gene flanked by loxP sites, and a 8.4-kb *Sph*I-*Sma*I fragment as the 3′ homologous sequences (see Fig. 1*A*). A PGK promoter-driven thymidine kinase (*tk*) gene was inserted upstream of the 5′ homologous sequences. J1 ES cells were transfected with the targeting construct, and G418 and gancyclovir double-resistant clones were analyzed by Southern blot to identify homologous recombinants (see Fig. 1*B*). Heterozygous mutant ES cell clones were grown in high G418 concentrations to select for homozygous mutant ES cell clones (25). The *PGK-neo* was deleted by transient expression of Cre in multiple heterozygous and homozygous mutant ES cell clones. Homozygous ES cells with or without the inserted *PGK-neo* were then transfected with a functionally assembled TCRβ vector (26).

Various mutant ES cells were injected into recombination-activation gene (RAG)-2-deficient blastocysts to generate chimeric mice (27). In addition, germline mutant mice were derived from a herterozygous mutant ES cell clone with *PGK-neo*. The inserted *PGK-neo* in some of these mice was then deleted by breeding germline mutant mice with a *cre* transgenic mouse (28). Germline mutant mice, with or without *PGK-neo*, were bred with TCRβ-transgenic mice (derived from the same TCRβ construct as used in the transfection) to obtain transgenic mutant mice. The names and genotypes of various chimeric and germline mutant mice are as depicted in Table I.

Abs and flow cytometry analyses

Abs specific to CD3, CD4, CD8, Thy-1.2, Vβ2, 4, 6, 7, 8.1/8.2, 9, 10, 12, 13, and 14 were direct conjugates from BD PharMingen (San Diego, CA). Single-cell suspensions were prepared from lymph nodes and thymi. A total of 5×10^5 cells were stained with the appropriate combination of Abs, and 10,000–20,000 live cells (propidium iodide negative) were collected for each sample using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). Data were analyzed using CellQuest software (BD Biosciences).

$CD44^+CD4^-CD8^-$ thymocytes were purified by complement-mediated lysis followed by cell sorting. A single-cell suspension of whole thymi was washed twice with PBS and resuspended in medium (HEPES-buffered RPMI 1640, supplemented with 5% FCS, L-glutamate, 2-ME, penicillin, and streptomycin) at 2×10^7 cells/ml. Cells were stained with Abs specific for CD4 and CD8 at 2.5 μg/ml for 1 h on ice, pelleted and washed with medium, and resuspended again at 2×10^7 cells/ml. Low-Tox-M Rabbit Complement (CL3051; Cedarlane Laboratories, Hornby, Ontario, Canada) was added at a 1/10 dilution, and the mixture was incubated for 1 h at 37°C with frequent mixing. Cells were then pelleted and washed with medium, and live cells were collected by Ficoll-Paque centrifugation. Cells were then stained with FITC-CD44 and PE-CD25 Abs, and $CD44^+$ cells were sorted by FACS.

Southern and Northern blot analyses

Following overnight digestion with proteinase K, DNA from tails and single-cell suspensions of lymph nodes or thymi were isolated by phenol/chloroform extraction and ethanol precipitation. For Southern blotting analysis, 10 μg of DNA was digested with the appropriate enzymes, fractionated on a 0.8% agarose gel, and transferred to ζ-probe filters. Filters were hybridized with ^{32}P-labeled probes and exposed to phosphorimaging screens. Total RNA was isolated from thymi using RNazol (Biotecx, Houston, TX), as per the manufacturer's instructions. For Northern blotting analysis, 10 μg of total RNA was fractionated on a 1.2% formaldehyde agarose gel and transferred to ζ-probe filters. The filters were hybridized with ^{32}P-labeled probes and exposed to phosphorimaging screens. Images were analyzed by ImageQuant (Molecular Dynamics, Sunnyvale, CA).

Table I. *Names and genotypes of various chimeric and germline mutant mice*

Name	Genotype
+/Vβ(neo)	Heterozygous Vβ insertion with the *PGK-neo*
Vβ/Vβ(neo)	Homozygous Vβ insertion with the *PGK-neo*
+/Vβ(Δneo)	Heterozygous Vβ insertion without the *PGK-neo*
Vβ/Vβ(Δneo)	Homozygous Vβ insertion without the *PGK-neo*
+/Vβ(neo);tg-5[a]	Heterozygous Vβ insertion with the *PGK-neo* and a TCRβ transgene
+/Vβ(Δneo);tg-5	Heterozygous Vβ insertion without the *PGK-neo* but with a TCRβ transgene
Vβ/Vβ(neo);tg-3	Homozygous Vβ insertion with the *PGK-neo* and a TCRβ transgene
Vβ/Vβ(Δneo);tg-2	Heterozygous Vβ insertion without the *PGK-neo* but with a TCRβ transgene
+/+;tg-5	Wild-type mice with a TCRβ transgene

[a] tg-5 represents the germline TCRβ-transgenic mouse strain; the others, indicated by different numbers, are chimeric mice generated with different ES cell clones carrying the same TCRβ transgene but at different integration sites.

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PCR and RT-PCR

Nested PCR for measuring Vβ13 to Dβ1Jβ1.1 rearrangement were performed in a 50-μl reaction containing 0.6 μg of lymph node DNA, 100 ng of each primer (1 and 4), 0.2 μM of each dNTP, 3.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl (pH 8.3 at 20°C), and 1 U *Taq* polymerase. Primary reactions were run for 12 cycles of 15 s at 94°C, 30 s at 61°C, and 2 min at 72°C. Next, 2 μl were transferred from the primary reactions to new tubes for secondary PCR that were performed with identical conditions, except with nested primers (2 and 5) and 18 cycles of amplification. Then, 25 μl of secondary PCR were electrophoresed on a 1.5% agarose gel, transferred to ζ-probe membranes, hybridized with ^{32}P-labeled Vβ13 cDNA probe, and exposed to phosphorimaging screens.

Seminested PCR for amplifying Vβ12 to Dβ1Jβ1.1 rearrangements were performed in a 50-μl reaction using the same buffers as above, except that primers 3 and 4 were used. Primary reactions were run for 12 cycles of 15 s at 94°C, 30 s at 61°C, and 2 min at 72°C. Next, 2 μl is transferred from the primary reactions to new tubes for secondary reactions that were performed with identical conditions, except with seminested primers (3 and 5) and 16 cycles of amplification. Then, 25 μl of secondary PCR were analyzed by Southern blotting as above using a Vβ12 cDNA probe.

Seminested PCR for amplifying Vβ13 to Dβ1 rearrangements were performed in a 50-μl reaction using 1.2 μg, DNA, primers 1 and 6, and the same buffer conditions as described above. Primary reactions were 25 cycles of 30 s at 94°C, 30 s at 61°C, 2 min at 72°C. Secondary reactions were done using 2 μl of the primary reactions and the same conditions as above except seminested primers (2 and 6) and 20 cycles of amplification. Then 10 μl of the secondary reactions were analyzed by Southern blotting as above using a Vβ13 cDNA probe.

Semiquantitative JAK3 PCR were done as previously described (15). Briefly, reactions were performed in a 50-μl reaction containing 50 ng of DNA, 100 ng of each primer (7 and 8), 0.2 μM of each dNTP, 2 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl (pH 8.3 at 20°C), and 1 U *Taq* polymerase. Primary reactions were run for 12 cycles of 15 s at 94°C, 30 s at 61°C, and 2 min at 72°C. Next, 2 μl were transferred from the primary reactions to new tubes for secondary PCR that were performed with identical conditions, except with seminested primers 7 and 9, and 25 cycles of amplification. Then, 25 μl of the secondary amplification were loaded on a 1.5% agarose gel and stained with ethidium bromide.

PCR for specifically amplifying Vβi to Dβ1Jβ1.1 rearrangements from thymic and lymph node DNA were performed using the Expand High Fidelity PCR System kit (Boehringer Mannheim, Mannheim, Germany) as per the manufacturer's instructions. A 50-μl PCR contained 0.6 μg DNA, 100 ng of primers 14 and 5, 0.4 μM of each dNTP, 4.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl (pH 8.3 at 20°C), and 1 U of enzyme. An initial heating of reaction mixture at 94°C for 3 min was followed by 10 cycles of 30 s at 94°C, 30 s at 63°C, and 3 min 45 s at 68°C, immediately followed by 20 cycles of 30 s at 94°C, 30 s at 63°C, and (3 min 45 s plus 20 s/cycle) at 68°C. Then 25 μl of PCR were analyzed by Southern blotting as above using a Vβ13 cDNA probe.

RT-PCR for assaying germline Vβ13 transcripts were performed using total RNA from thymi. Reactions were performed in 50 μl using the One-Tube Titan kit (Boehringer Mannheim) as per the manufacturer's instructions. A cDNA synthesis reaction was performed at 50°C for 30 min using 30 ng total RNA, 1 ng of each primer (10 and 11), and 3.5 mM $MgCl_2$. Amplification of cDNA was done in the same reaction tube, using the following conditions: 30 s at 94°C, 30 s at 60°C, 2 min at 72°C, for 22 cycles. Then, 25 μl of reactions were analyzed by Southern blotting as above using a Vβ13 cDNA probe.

RT-PCR for assaying immature β-actin transcripts (29) were performed on total RNA isolated from thymi using the same reaction conditions as above except using primers 12 and 13. Then 25 μl of reactions were electrophoresed on a 1.0% gel and stained with ethidium bromide.

Primer sequences are as follows: 1, 5′-CTGCCATGGGCACCAGGCTTCTTG; 2, 5′-GGCACCAGGCTTCTTGGCTGGGCAG; 3, 5′-GCTGGAGTTACCCAGACACCC; 4, 5′-AGATACTCGAATATGGACACGGAG; 5, 5′-TGGACACGGAGGACATGCTTTGTC; 6, 5′-CAATCTTGGCCTAGCAGGCTGCAG; 7, 5′-CCTCTCAGACCCCACACCTGGCATC; 8, 5′-CCATAGCTGACTCCCCGGTACTTG; 9, 5′-ACGATGAAGTCGCTGTGCAGAGCCTTA; 10, 5′-TCCTTGACACAGTACTGTCTGAAGC; 11, 5′-CTCTGGATACACGCAGCATGGCCT; 12, 5′-CCTAAGGCCAACCGTGAAAAG; 13, 5′-TCTTCATGGTGCTAGGAGCCA; 14, 5′-CACTCGCTGCATCCTACACATAGCGCTC.

Results

Generation of mice with targeted insertion of Vβ13 gene segment

At the murine TCRβ locus, Dβ and Jβ gene segments are clustered together and are within 18 kb of the Eβ enhancer. In contrast, Vβ gene segments are dispersed over 250 kb and are at least 340 kb away from Dβ and Eβ, with the exception of Vβ14, which is only 5 kb away from the Eβ at the 3′ end of the locus (Fig. 1) (30, 31). We inserted a copy of the Vβ13 gene segment, called Vβi, together with 3.6-kb 5′ and 0.7-kb 3′ flanking sequences 6.8 kb upstream of the Dβ1 at the TCRβ locus through homologous recombination in ES cells (Fig. 1 and *Materials and Methods*). Compared with the natural Vβ13 gene segment, referred to as Vβe, which is 470 kb 5′ of Dβ1, Vβi is ~70 times closer to Dβ and Eβ than Vβe on the linear chromosomal DNA. Heterozygous and homozygous mutant ES cells in the presence or absence of the *PGK-neo* cassette were differentiated into mature T cells by RAG-2-deficient blastocyst complementation (27). Germline mutant mice

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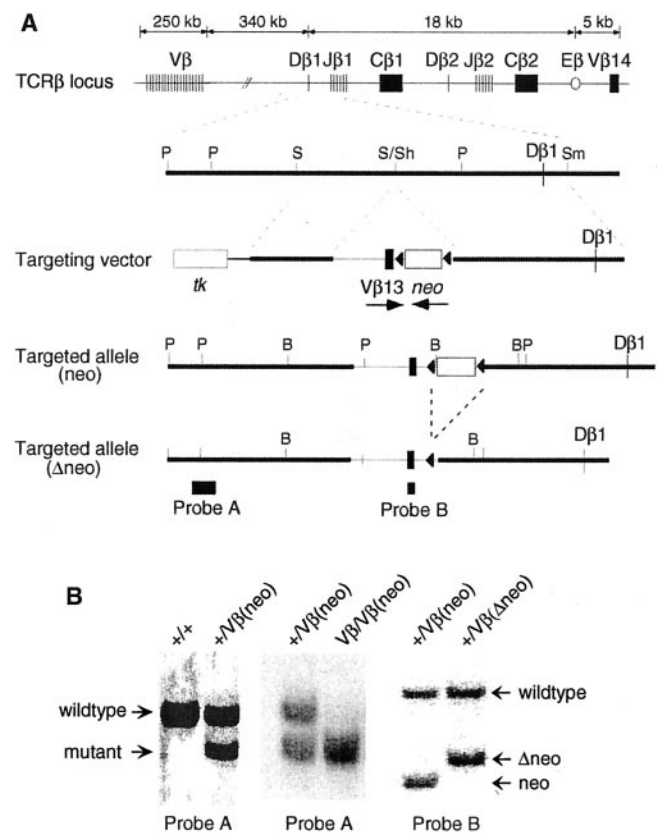

FIGURE 1. Targeted insertion of the Vβ13 gene segment. *A,* Schematic diagrams of the murine TCRβ locus (not to scale), targeting vector, and targeted allele before (neo) and after (Δneo) Cre-mediated deletion of the *PGK-neo*. Arrows flanking *PGK-neo* represent loxP sites. Transcriptional orientations of Vβ13 and *PGK-neo* are indicated. Probe A is a 1.5-kb *Hin*dIII-*Eco*RI fragment; probe B is a 450-bp cDNA containing Vβ13. P, *Pvu*II; S, *Spe*I; Sh, *Sph*I; Sm, *Sma*I; B, *Bam*HI. *B,* Southern blot analysis for identifying homologous recombinant ES cell clones before and after *PGK-neo* deletion. DNA from ES cell clones was digested with *Pvu*II and hybridized with probe A. The wild-type allele gives rise to a 11.5-kb fragment, and the targeted allele gives rise to a 9.6-kb fragment. Probe A also hybridized with a 1.8-kb fragment from both wild-type and targeted alleles (data not shown). After Cre-mediated deletion, DNA from mutant ES cell clones was digested with *Bam*HI and hybridized with probe B. The targeted allele with *PGK-neo* gives rise to a 8.6-kb fragment, and the targeted allele with deleted *PGK-neo* gives rise to a 10.6-kb fragment.

with or without the *PGK-neo* were also derived. There was no difference between the results obtained from chimeric mice derived from targeted ES cells and germline mutant mice. Therefore, no distinction is made whether analyzed mice were chimeras or germline mutant mice, unless necessary. A partial list of generated mice and their genotypes is detailed in *Materials and Methods*.

Frequency of Vβi rearrangement

The relative usage of Vβi in recombination was estimated by comparing the percentages of Vβ13-expressing T cells between wild-type and mutant mice. Lymph node cells were stained with an Ab specific to the pan-T cell marker Thy-1.2 and Abs to Vβ13, Vβ6, or Vβ14, then analyzed by flow cytometry. In wild-type (+/+) mice, an average of 2.3% of Thy-1.2$^+$ T cells expressed Vβ13 (Fig. 2 and Table II). In homozygous mutant mice without the *PGK-neo* (Vβ/Vβ(Δneo)), ~4.7% T cells were Vβ13 positive. The frequencies of Vβ6, Vβ14, and other Vβ-expressing T cells were similar between wild-type and mutant mice (Fig. 2 and data not shown), indicating that Vβi did not significantly affect the rearrangement and expression of the endogenous Vβ gene segments. Because T cells from Vβ/Vβ(Δneo) mice had four copies of the Vβ13 gene segment compared with two in wild-type T cells, the increase in percentages of Vβ13-expressing T cells correlated with the copy numbers of the Vβ13 gene segment. Thus, despite its proximity to Dβ and Eβ, Vβi appears to be used at the same frequency as Vβe in mature T cells.

In contrast, the frequencies of Vβ13-expressing T cells were markedly increased when *PGK-neo* was left at the insertion site. On average, 11.0 and 14.0% of lymph node T cells were Vβ13 positive in heterozygous +/Vβ(neo) and homozygous Vβ/Vβ(neo) mice, respectively (Fig. 2 and Table II), indicating there is no intrinsic selection against Vβ13-expressing T cells. Although the frequency of Vβ13-expressing T cells was substantially increased, the frequencies of other Vβ-expressing T cells were only slightly and variably changed (Fig. 2 and data not shown, see also Fig. 4*B*). Assuming that Vβe rearranged at approximately the same frequency (~1.15% per allele) in the presence of *PGK-neo*, the frequency of Vβi rearrangement was 8.7 and 10.7% (~5.35% per allele) in heterozygous and homozygous mutant mice, respectively, an increase of ~5- to 7-fold. *PGK-neo* was constitutively transcribed (data not shown) and was deleted upon Vβi rearrangement. As *PGK-neo* does not appear to have an intrinsic property in promoting DNA recombination (see *Discussion*), the localized *neo* transcription and its associated chromatin changes probably promotes Vβi rearrangement, resulting in increased percentages of Vβ13-expressing T cells.

Table II. *Percentages of Vβ13$^+$ T cells in lymph nodes of various types of mice*[a]

+/+	Vβ/Vβ(Δneo)	+/Vβ(neo)	Vβ/Vβ(neo)
2.0	4.8	11.4	13.5
2.2	5.0	12.3	13.8
2.2	4.7	10.7	12.3
2.3	4.7	11.5	12.6
2.3	4.2	10.3	17.2
2.3	4.2	10.5	14.4
2.3	4.6	10.4	14.2
2.4		10.8	
2.4		10.2	
2.5		9.9	
2.5		12.7	
2.3 ± 0.14[b]	4.7 ± 0.3	11.0 ± 0.9	14.0 ± 1.6

[a] Lymph node cells were stained with anti-Thy-1.2 and anti-Vβ13 Abs. Percentages of Vβ13$^+$ T cells were calculated as the percentages of Thy-1.2$^+$ cells. Approximately 3% of T cells in +/Vβ(Δ*neo*) mice were Vβ13 positive (not shown).
[b] Mean ± SD.

The frequencies of Vβi rearrangement in the presence or absence of *PGK-neo* were confirmed by semiquantitative PCR analyses of Vβ13 to Dβ1Jβ1.1 rearrangement in DNA from lymph node cells. Consistent with the results by flow cytometry, the level of PCR product was substantially higher (~5 fold) in +/Vβ(neo) mice than in wild-type and +/Vβ(Δneo) mice, and the level of the amplified product was similar between +/Vβ(Δneo) mice and wild-type mice (Fig. 3*A*, *lanes 1–3*). Identical results were also obtained for Vβ13 to DβJβ1.1 rearrangement in DNA from thymus (Fig. 3*B*). PCR assays specific for the Vβi-Dβ1Jβ1.1 rearrangement showed that the expected 4.5-kb product was detected only in thymic and lymph node DNA from targeted mice, but not wild-type mice (Fig. 3*C*). Because the large size of the PCR product precluded efficient amplification, the long-range PCR was not quantitative. Nonetheless, taken together, these data show that in the absence of *PGK-neo*, Vβi is rearranged at the same frequency as Vβe, but its frequency of rearrangement is much higher than that of Vβe in the presence of *PGK-neo*.

Allelic exclusion of the Vβi gene segment

To investigate Vβi allelic exclusion, we examined the frequencies of Vβ13-expressing T cells in the presence of a functionally assembled TCRβ transgene. The TCRβ transgene expressed Vβ8.2, and the extent of its expression on mature T cells was monitored by flow cytometry using anti-CD3 and anti-Vβ8.1/8.2 Abs. In wild-type (+/+) mice, ~18% of CD3$^+$ T cells expressed Vβ8.1/8.2. In TCRβ (tg-5)-transgenic mice on the +/+, +/Vβ(Δneo), or +/Vβ(neo) background, over 99% of T cells expressed the TCRβ transgene (Fig. 4*A*). In these same mice, the percentage of Vβ13-expressing T cells was decreased at least 18-fold (Fig. 4, *A* and *B*). Similar folds of decrease in Vβ4-, Vβ6-, or Vβ12-expressing T cells were also observed. Thus, the expression of the TCRβ (tg-5) transgene appears to exclude the expression of the endogenous Vβs as well as Vβi.

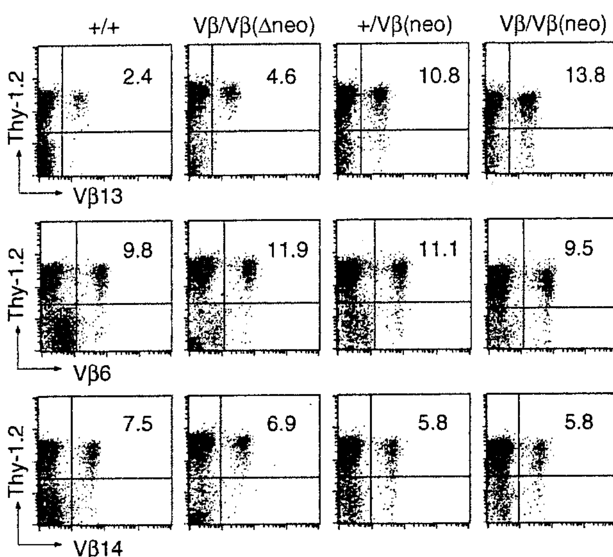

FIGURE 2. Flow cytometry analysis of Vβ expression on lymph node T cells. Lymph node cells from wild-type and mutant mice were stained with anti-Thy-1.2 and anti-Vβ Abs, followed by flow cytometry. The numbers indicate the percentages of T cells (Thy-1.2$^+$) that express the specific Vβ.

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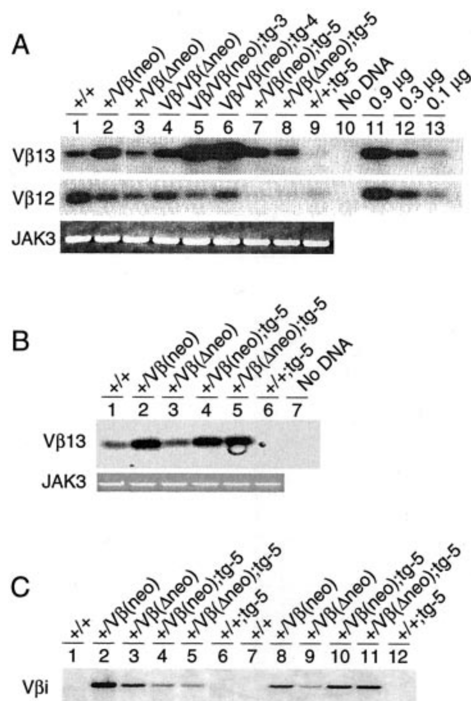

FIGURE 3. PCR assays for Vβ rearrangements. *A,* Comparison of the levels of Vβ to Dβ1Jβ1.1 rearrangements among various types of mice. PCR products representing Vβ13 or Vβ12 to Dβ1Jβ1.1 rearrangements were detected with a Vβ13 or a Vβ12 cDNA probe, respectively. *Lanes 11–13* are PCR using 0.9, 0.3, and 0.1 μg, respectively, of input DNA from +/Vβ(*neo*) mice to show the linearity of the PCR. PCR amplification of JAK3 shows the relative amount and quality of DNA for each sample. For germline mutant mice, either with or without the TCRβ transgene (*lanes 1–3* and *7–9*), similar results were obtained from at least two independent mice assayed in two different PCR. For chimeric transgenic mice (*lanes 4–6*), similar results were obtained from two independent PCR. *B,* PCR analysis of Vβ13 to Dβ1Jβ1.1 rearrangements in thymic DNA of various types of mice. *C,* PCR analysis of Vβi to Dβ1Jβ1.1 rearrangements in thymic (*lanes 7–12*) and lymph node (*lanes 1–6*) DNA of various types of mice.

The same TCRβ transgene was also transfected into Vβ/Vβ(Δneo) and Vβ/Vβ(neo) ES cells, and allelic exclusion of Vβi expression was analyzed in chimeras derived from ES cell clones. Like tg-5-transgenic mice, Vβ13-expressing T cells were reduced ~18-fold (from 4.7 to 0.26%) in chimeras generated with Vβ/Vβ(Δneo);tg-2 and with Vβ/Vβ(Δneo);tg-1 ES cell clones (Fig. 4, *A* and *B*). However, Vβi exclusion was leaky in chimeras generated with Vβ/Vβ(neo);tg-3 and Vβ/Vβ(neo);tg-4 ES cell clones (in the presence of the *neo* cassette). For example, in Vβ/Vβ(neo); tg-3 chimeras the TCRβ transgene was expressed on 97% of T cells and Vβ6-expressing T cells were decreased ~9-fold, but Vβ13-expressing T cells were decreased only 3-fold (from 14 to 4.3%). In fact, 3.7% of T cells expressed both Vβ13 and the Vβ8.2 transgene (Fig. 4*A*). The leaky Vβi exclusion could be caused by the presence of *PGK-neo* and/or by different levels of TCRβ transgene expression due to different copies of the transgene that were integrated at different sites. Nevertheless, even in the presence of *PGK-neo,* Vβi expression is excluded in over 95% of total T cells by the TCRβ transgene.

To verify Vβi allelic exclusion at the DNA level, Vβ13 rearrangement was assayed by PCR in lymph node DNA of various transgenic mutant mice. In +/+;tg-5 mice, PCR product corresponding to Vβ13 to Dβ1Jβ1.1 rearrangement was barely detectable (Fig. 3*A*, *lane 9*), indicating allelic exclusion of the endogenous Vβ13 and correlating with the results obtained by flow cytometry. Unexpectedly, a slightly higher levels of Vβ13 rearrangement were detected in +/Vβ(Δneo);tg-5 mice than in wild-type or +/Vβ(Δneo) mice (Fig. 3*A*, *lanes 1*, *3*, and *8*), although the mutant mice had <0.13% of Vβ13-expressing T cells as compared with 2.3% in wild-type mice and 3.5% in +/Vβ(Δneo) mice (Table II). Similar levels of Vβ13 rearrangement were also detected in thymocyte DNA from the three types of mice (Fig. 3*B*). The loss of exclusion was specific for Vβi (Vβ13) because rearrangement of Vβ12 was undetectable in the same DNA from +/Vβ(Δneo); tg-5 mice (Fig. 3*A*, *lanes 8* and *9*). In the presence of *PGK-neo* as in +/Vβ(neo);tg-5 mice, even higher levels of Vβ13 rearrangement were detected (Fig. 3*A*, *lanes 1* and *7–9*). Particularly, in Vβ/Vβ(neo);tg-3 and Vβ/Vβ(neo);tg-4 chimeras, where allelic exclusion was leaky as shown by flow cytometry (Fig. 4) and by the presence of Vβ12 rearrangement at the DNA level (Fig. 3*A*), the levels of Vβ13 rearrangement were ~10- to 20-fold higher than those in wild-type mice (Fig. 3*A*, *lanes 1*, *5*, and *6*). Together, these data show that while rearrangements of endogenous Vβ are inhibited by the presence of a TCRβ transgene, Vβi continues to rearrange at a similar level (~3–5%) as if in the absence of the transgene. However, the majority of the rearranged Vβi is not expressed on the cell surface.

To probe the discrepancy between the levels of Vβ13 rearrangement detected by PCR and by cell surface Vβ13 staining, we assayed for Vβ13-containing transcripts in total thymic RNA by Northern blotting analysis. The 1.3-kb mature transcript was readily detected in wild-type, +/Vβ(neo), and +/Vβ(Δneo) mice (Fig. 5*A*, *lanes 1–3*), with levels of the transcript corresponding to the percentages of T cells that expressed Vβ13 in these mice. As expected, in the presence of the TCRβ transgene (tg-5), no mature transcript was detected in wild-type or mutant mice either in the presence or absence of *PGK-neo* (Fig. 5*A*, *lanes 4–6*). Germline Vβ13 transcript, which is around 1.0 kb, was not detected on the Northern blot, but was readily detected by RT-PCR, even in the presence of the TCRβ transgene (Fig. 5*B*). These results show that, in the presence of the TCRβ transgene, most rearranged Vβi is not highly transcribed and therefore not expressed on the cell surface.

Timing of Vβi rearrangement

As shown above, in the absence of *PGK-neo* and the TCRβ transgene, Vβi rearranges and expresses like Vβe; however, Vβi rearrangement is not inhibited to the same extent as the endogenous Vβs by the TCRβ transgene. To determine whether the leaky Vβi allelic exclusion results from an earlier onset of Vβi rearrangement, we assayed the levels Vβ13 rearrangement in $CD4^-CD8^-CD44^+$ thymocytes. Normally, $CD4^-CD8^-$ (double negative or DN) thymocytes progress from $CD44^+CD25^-$ to $CD44^+CD25^+$, then to $CD44^-CD25^+$, and finally to $CD44^-CD25^-$ phenotype. TCRβ gene rearrangement occurs predominantly at the $CD44^-CD25^+$ stage. If the onset of Vβi rearrangement occurs earlier, one would expect to detect higher levels of Vβ13 rearrangement in $CD44^+$ fraction of DN thymocytes from +/Vβ(neo) mice as compared with wild-type mice. Thus, $CD44^+$ DN thymocytes were purified from wild-type and +/Vβ(neo) mice (97% $CD44^+$), and the levels of Vβ13 to Dβ1Jβ1.1 rearrangement were assayed by semiquantitative PCR (Fig. 6*A*). Quantification of the intensity of Vβ13 PCR product and normalization of the input DNA by JAK3 PCR amplification showed that ~10% more Vβ13 rearrangement was detected in +/Vβ(neo) DNA than in wild-type DNA (Fig. 6*A*, *lanes 5* and *6*). Compared with the levels of Vβ13 rearrangements in total wild-type thymus, the level of Vβ13 rearrangement in the $CD44^+$ fraction was ~8- to 10-fold lower (Fig. 6*A*, *lanes 1–6*). Because the

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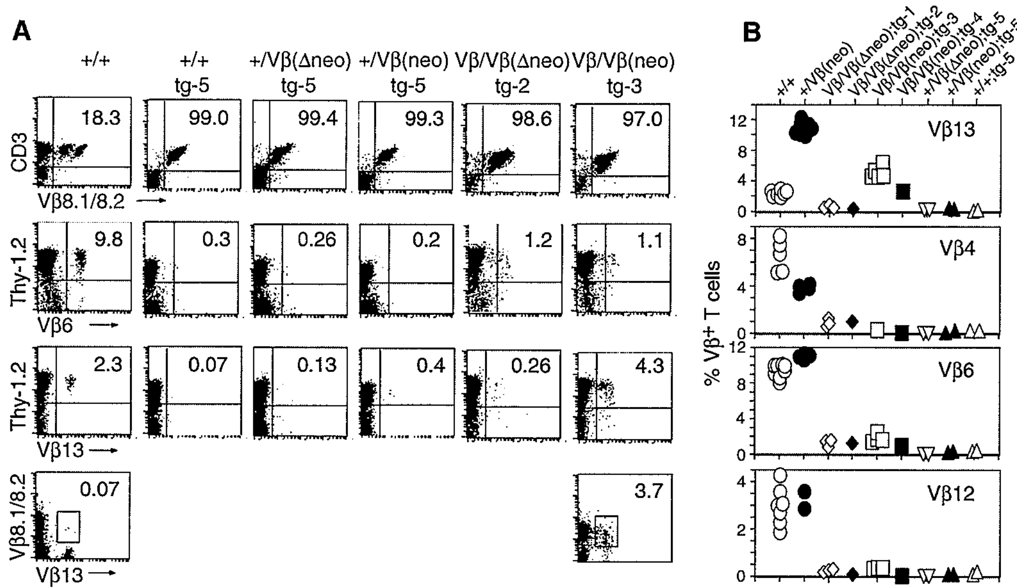

FIGURE 4. Analysis of allelic exclusion by flow cytometry. *A,* Flow cytometry analysis of Vβ expression on lymph node T cells. Lymph node cells were stained with anti-CD3 and anti-Vβ8.1/8.2, or anti-Thy-1.2 and anti-Vβ6 or Vβ13 Abs, followed by flow cytometry. The numbers indicate the percentages of T cells ($CD3^+$ or Thy-1.2^+) that express the specific Vβ. In the FACS plots showing double staining of Vβ8.1/8.2 and Vβ13, the numbers indicate the percentages of Vβ13 cells that are also Vβ8.1/8.2^+. *B,* Summary of percentages of Vβ-expressing T cells in lymph nodes of various types of mice. Lymph node cells were assayed as in *A*. TCRβ transgenes, tg-1 and tg-4, were expressed on 95.5 and 99% of T cells, respectively. Each symbol represents an individual mouse.

level of Vβ13 rearrangement in wild-type thymus is ~2.3% of the total Vβ rearrangement, the overall level of Vβ13 rearrangement in the $CD44^+$ fraction of +/Vβ(neo) thymus is low. Together, these results demonstrate that Vβi is not significantly recombined earlier during T cell development.

We also assayed for the presence of Vβ13 to Dβ1 rearrangement, before Dβ1-Jβ rearrangement, in total thymic DNA from various types of mice by PCR. As expected, virtually no Vβ13Dβ1 rearrangement was detected in wild-type mice or in TCRβ-transgenic mice on a wild-type background (Fig. 6*B*, *lanes 1* and *6*). In

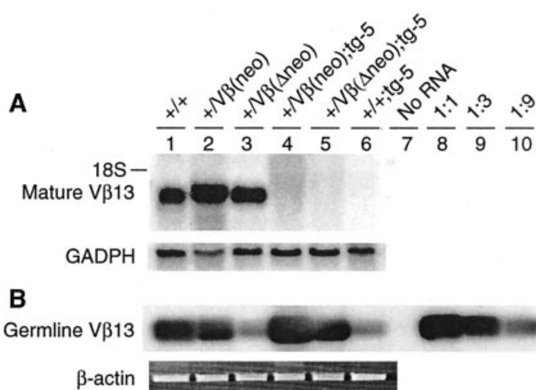

FIGURE 5. Vβ13 transcription in thymocytes. *A,* Northern blot analysis of Vβ13 transcripts. Ten micrograms of total RNA from thymocytes of wild-type and mutant mice were fractionated, and the filter was hybridized with a Vβ13 cDNA probe. The filter was stripped and rehybridized with a GAPDH probe for monitoring the relative amount of RNA in each sample. The position of 18S RNA is indicated. *B,* RT-PCR analysis of germline Vβ13 transcripts. Total RNA from thymocytes was assayed for Vβ13 germline transcripts by RT-PCR. PCR product was verified by hybridization with a Vβ13 cDNA probe. *Lanes 8–10* are PCR using different dilutions of input RNA from +/Vβ(neo) mice to show the linearity of PCR. RT-PCR for β-actin transcripts was used to estimate the relative amount of RNA in each sample.

contrast, Vβ13Dβ1 rearrangements were detected in +/Vβ(neo) and +/Vβ(Δneo) mice, even in the presence of the TCRβ transgene (Fig. 6*B*, *lanes 2–5*). Vβ13 to Dβ1 rearrangements were authentic as shown by the presence of N region nucleotides and nucleotide deletion in 13 independent PCR products from +/Vβ(neo) mice (Fig. 6*C*), suggesting that V to D recombination is mechanistically normal.

To quantify the level of Vβ13Dβ1 rearrangement, we used as comparison the total thymic DNA from $J\beta1^{M2/\omega}$ mutant mice, in which the Dβ2-Jβ2-Cβ2 region of the TCRβ locus was deleted (32). In $J\beta1^{M2/\omega}$ mice, the ω allele undergoes both Dβ1-Jβ1 and Vβ-Dβ1Jβ1 rearrangements, whereas the M2 allele undergoes only Vβ-Dβ1 rearrangement because the 3′ Dβ1 RSS was mutated. It was previously shown that the M2 allele undergoes diverse Vβ to Dβ1 rearrangement at a frequency of 21% (32). Assuming that usage of Vβ13 in $J\beta1^{M2/\omega}$ mice is comparable to that in wild-type mice (2.3%), because the level of Vβ13Dβ1 rearrangement in our mutant mice is approximately one-third the level of Vβ13Dβ1 rearrangement observed in $J\beta1^{M2/\omega}$ mice, the percentage of Vβi allele undergoing Vβ13Dβ1 rearrangement is 0.16% $[(0.21)(0.023)(0.33) \times 100]$. Thus, the steady-state level of VβiDβ1 rearrangement in total thymocytes is at least 10 time lower than Vβ13DβJβ rearrangement in wild-type mice.

Discussion

Position vs distance in V(D)J recombination

Recombination of Vβ gene segments is controlled in terms of frequency, timing, order, and allelic exclusion. Insertion of the Vβ13 gene segment 6.8 kb upstream of the Dβ1 not only dramatically shortens the distance between the Vβ and the Dβ1 gene segment but also may have removed the Vβ from regulation by *cis*-regulatory elements naturally present in the Vβ region (position effect). A role of distance between gene segments and/or their position in the locus on V(D)J recombination was initially suggested by observations at the murine IgH locus. It was found that initial DJ_H

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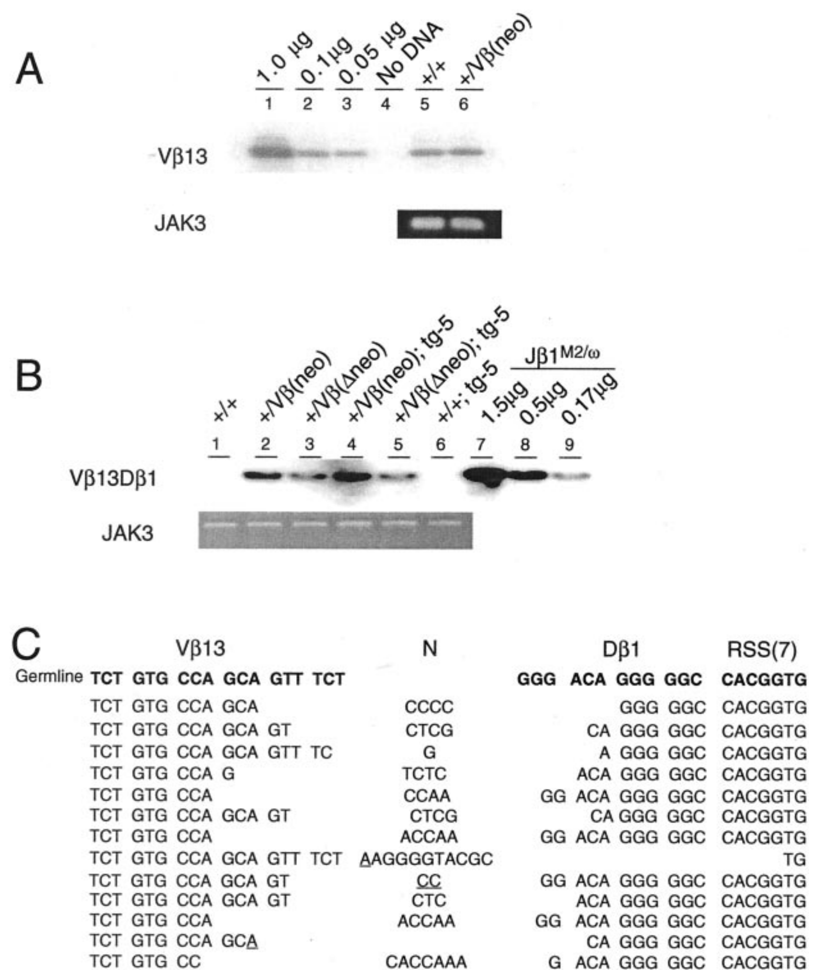

FIGURE 6. Analysis of the timing and the order of Vβ13 rearrangements. *A,* Comparison of Vβ13 to Dβ1Jβ1.1 rearrangements in total and purified CD44$^+$ DN thymocytes. CD44$^+$ DN thymocytes from wild-type and +/Vβ(neo) mice were purified by complement-mediated lysis of CD4$^+$, CD8$^+$, and CD4$^+$CD8$^+$ cells followed by cell sorting for CD44$^+$ cells (>97% CD44$^+$). DNA (1.0 μg) isolated from purified CD44$^+$ DN thymocytes was assayed for Vβ13 to Dβ1Jβ1.1 rearrangement (*lanes 5* and *6*) and JAK3 as in Fig. 3*A*. Different amounts of total thymic DNA (1.0, 0.1, and 0.05 μg) from a wild-type mouse was assayed (*lanes 1–3*) to estimate the level of rearrangements in CD44$^+$ cells. *B,* Comparison of the levels of Vβ13 to Dβ1 rearrangement in various types of mice. DNA from thymocytes (1.2 μg) was amplified for Vβ13 to Dβ1 rearrangements by two rounds of PCR. Different amounts of thymocyte DNA from Jβ1$^{M2/\omega}$ mice were amplified to estimate the level of Vβ13 to Dβ1 rearrangements (*lanes 7–9*). Specific PCR product was detected by hybridization with a Vβ13 probe. PCR amplification of JAK3 shows the relative amount of DNA for each sample. *C,* Sequence analysis of Vβ13-Dβ1 rearrangements. Vβ13-Dβ1 rearrangements from +/Vβ(neo) mice were subcloned and sequenced. Overlapping nucleotides that could be encoded by either germline segment or P nucleotides are underlined.

rearrangements preferentially use the D segments proximal to J_H while the rearrangements of the more distal D segments occur through secondary rearrangements (33, 34). Similarly, V_H segments that are proximal to J_H are preferentially used in VDJ recombination during fetal B cell development (35–37) and tend to escape allelic exclusion in Igμ-transgenic mice (38). Recently, it was found that IL-7R was preferentially required for the rearrangement of the J_H-distal but not the J_H-proximal V_H gene segments (39). Because IL-7R-mediated signaling probably promotes V_H rearrangement by modulating recombination accessibility (40, 41), this finding implies that different *cis*-regulatory elements may control recombination accessibility of the J_H-distal and J_H-proximal V_H gene segments (position effect).

If distance between gene segments affects their frequency of recombination, one would expect that Vβi would be preferentially used in TCRβ recombination in our mutant mice. However, our results clearly show that, in the absence of *PGK-neo*, Vβi is rearranged at the same frequency as the endogenous copy (Figs. 2 and 3 and Table II). The failure to detect higher levels of Vβi rearrangement and expression is not because an initial Vβi rearrangement is deleted by a subsequent rearrangement of an upstream Vβ to a downstream DβJβ or because there is an intrinsic selection against Vβ13-expressing T cells. Much higher levels of Vβ13 rearrangement and expression were detected if *PGK-neo* was left at the insertion site. Our results are consistent with observations that recombination frequencies of Vβ gene segments are not correlated with their distances to the Dβs in the TCRβ locus (3). For example, the Vβ14 gene segment is within 25 kb of the Dβ gene segments but is not most frequently recombined and expressed (3). In contrast, the Vβ8.2 gene segment, which is used with the highest frequency in TCRβ rearrangement, is not the most proximal to the Dβ-Jβ region (30, 31). Vβ8.2 is highly transcribed during early T lymphocyte development (42, 43), suggesting that local accessibility of V gene segments is probably more important in regulating recombination frequency (see below). In our targeted insertion, although we cannot exclude unequivocally a role of distance between Vβi and Dβ in Vβi rearrangement, our results are consistent with the interpretation that position of Vβ gene segments in the locus, and therefore their control by *cis*-regulatory elements, influences the Vβ gene rearrangement.

Local regulation of Vβ recombination frequency

The fact that Vβi recombines at the same frequency as the endogenous copy suggests that the inserted DNA fragment contains the necessary *cis*-regulatory elements for regulating the frequency of Vβ13 rearrangement. A potential *cis*-regulatory element in the inserted fragment is the Vβ promoter. Although Vβ promoters have not been shown to promote Vβ rearrangement, *cis* elements upstream of the Vγ gene segment, corresponding to the likely promoter, control the timing of Vγ rearrangement during development (44). Consistent with the promoter control of Vβ recombination frequency, the presence of the *PGK-neo* transcriptional cassette at the insertion site resulted in a 5- to 7-fold increase in Vβi usage in recombination (Fig. 3 and Table II). The stimulating effect of *PGK-neo* on Vβi rearrangement is in contrast to the inhibitory effect of the *neo* cassette on recombination when inserted into IgH and Igκ loci. Insertion of a *PMC1neo* downstream of Igκ intronic enhancer severely blocked Vκ to Jκ rearrangement upstream (45).

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Insertion of *PGK-neo* in between the 3′ IgH enhancer and constant region exons blocks class switch recombination to the constant genes 5′ of the *neo* cassette (46). As *PGK-neo* does not have an intrinsic property in promoting recombination, its effect on Vβi rearrangement likely reflects the mechanisms by which Vβ gene segments are normally targeted for recombination. The neo transcription is driven by a constitutively active PGK promoter, and the entire cassette is deleted after Vβi recombination. Recent studies have shown that transcriptional coactivators possess histone acetyltransferase activity (47–49) and histone acetylation is tightly correlated with V(D)J recombination accessibility (50). The transcription process itself also leads to changes in DNA-nucleosome interaction (51). Together, these findings suggest that chromatin changes, mediated by local *cis*-regulatory elements, probably determine Vβ recombination frequency.

Why is Vβi not recombined more frequently as its promoter is much closer to Eβ? An explanation is suggested by recent findings on the extent of Eβ and PDβ1 in controlling the chromatin structure of the TCRβ locus. We have shown previously that three DNase I-hypersensitive sites are present within 3 kb of Dβ1 gene segment (19) and the site immediately upstream of Dβ1 corresponds to the PDβ1 promoter (13, 14). In the presence of PDβ1 promoter, the promoter region as well as the downstream Dβ1-Jβ1 region are hypomethylated in developing T cells, whereas the upstream region including at least one DNase I-hypersensitive site is hypermethylated (16). Deletion of PDβ1 alone or plus the two upstream hypersensitive sites results in the invasion of hypermethylation into the downstream Dβ1-Jβ1 region. These findings suggest that there is probably a boundary between the inaccessible upstream region and the accessible Dβ1-Jβ1 region. Consistent with this interpretation, deletion of the Eβ resulted in the hypermethylation, histone hypoacetylation, resistance to endonuclease treatment, and transcriptional silencing of the Dβ-Jβ region, whereas no significant effect was observed in the Vβ region or 5 kb upstream of the Dβ1 gene segment (10). Thus, the chromosomal domain regulated by Eβ appears to be limited to the Dβ-Jβ region. Although Vβi is much closer to the Dβ1 gene segment and Eβ enhancer, it probably still resides within a relatively inaccessible region and outside the Eβ-regulatory domain, accounting for the absence of a higher level of Vβi rearrangement.

Long-range regulation of Vβ allelic exclusion

We found that Vβi was rearranged at a similar level in the absence or presence of a TCRβ transgene (Fig. 3), indicating that Vβi is no longer subject to allelic exclusion. The lack of Vβi allelic exclusion cannot be attributed significantly to an earlier onset of Vβi rearrangement because there is only a slight increase of Vβ13 rearrangement at the $CD44^{+}$ DN stage (Fig. 6). However, the presence of Vβ to Dβ rearrangement before Dβ-Jβ rearrangement may have contributed to the lack of Vβi allelic exclusion (Fig. 6). Because the Dβ-Jβ region remains accessible to the recombinase in double-positive thymocytes (15), VβDβ rearrangements likely undergo further VβDβ-Jβ rearrangements when the recombinase is re-expressed in double-positive thymocytes for TCRα rearrangement. Although 0.16% of VβDβ rearrangement is relatively low as compared with 2–5% Vβi rearrangement in the presence of a TCRβ transgene, the amount represents the steady-state level but does not take into account the rate of VβDβ generation and the rate of conversion to VβDβJβ. Based on these considerations, VβDβ rearrangement before DβJβ rearrangement probably has contributed to the Vβi rearrangement in the presence of a TCRβ transgene, but whether it can account for all Vβi rearrangement is not clear.

As discussed above, the inserted DNA fragment contains *cis*-regulatory elements for determining the frequency of Vβ13 rearrangement. The similar level of Vβi rearrangement in the presence of a TCRβ transgene suggests that the same *cis* elements are insufficient for mediating Vβi allelic exclusion. There are likely other *cis*-regulatory element in the Vβ region that normally mediate Vβ allelic exclusion, and insertion of the Vβ13 gene segment in the proximity of Dβ-Jβ region may have moved the Vβi outside the regulatory range of these additional *cis*-regulatory elements. It is possible that each Vβ has its own *cis* elements for mediating allelic exclusion and these elements happen not to be included in the inserted fragment. However, it seems more likely that a few Vβs or all Vβs share common *cis* elements for allelic exclusion (see *Introduction*). While the nature and location of these *cis*-regulatory elements are unknown, the continuous Vβi rearrangement at a similar frequency in the presence of a TCRβ transgene strongly suggests that the frequency and allelic exclusion of Vβ rearrangement is regulated by distinct *cis*-regulatory elements.

Interestingly, most of the rearranged Vβi was not expressed on the cell surface in the presence of a TCRβ transgene (Figs. 4 and 5). T cells have been shown to be capable of expressing two different TCRβ-chains simultaneously (52). Similarly, in our mutant mice, some T cells expressed both Vβ13 and Vβ8.2 (Fig. 4), suggesting that T cells expressing Vβ13 and Vβ8.2 are not intrinsically selected against. The lack of cell surface expression of the rearranged Vβi in the presence of a TCRβ transgene is because the rearranged Vβi is not highly transcribed (Fig. 5). During normal thymocyte development, transcription of the rearranged allele is up-regulated following recombination (53). This apparently did not occur in most of the developing T cells in which Vβi underwent rearrangement in the presence of a TCRβ transgene. While further studies are required to elucidate the mechanism of this regulation, the present findings clearly show that allelic exclusion can be mediated functionally by inhibiting transcription of a rearranged gene segment.

Regulation of the timing and order of Vβ rearrangement

In addition, we found a low level of Vβi to Dβ rearrangement occurring before Dβ-Jβ rearrangement and a small increase of Vβ rearrangement in $CD44^{+}$ DN thymocytes in the mutant mice as compared with the wild-type mice. Recently, it was shown that the 5′ RSS of Dβ1 plays an important role in the ordered DβJβ before VβDβJβ rearrangements (54). Although our findings could result from the close proximity of Vβi to the Dβ-Jβ region, we favor the notion that the Vβi is removed from the regulation by additional *cis*-regulatory elements normally present in the Vβ region. In this scenario, the significant increase of Vβi to Dβ joining in mutant mice would suggest that these additional *cis*-regulatory elements may also contribute to the ordered TCRβ rearrangement.

Acknowledgments

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