

# Gene placement and competition control T cell receptor $\gamma$ variable region gene rearrangement

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The production of distinct sets of T cell receptor (TCR)  $\gamma\delta^+$  T cells occurs in an ordered fashion in thymic development. The  $V\gamma 3$  and  $V\gamma 4$  genes, located downstream in the TCR $\gamma$   $C\gamma 1$  gene cluster, are expressed by the earliest waves of developing TCR $\gamma\delta^+$  T cells in the fetal thymus, destined for intraepithelial locations. Upstream  $V\gamma 2$  and  $V\gamma 5$  genes are expressed in later waves in the adult and constitute most TCR $\gamma\delta^+$  T cells in secondary lymphoid tissue. This developmental pattern is caused in part by a preference for rearrangements of the downstream  $V\gamma 3$  and  $V\gamma 4$  genes in the early fetal stage, which switches to a preference for rearrangements of the upstream  $V\gamma 2$  and  $V\gamma 5$  gene rearrangements in the adult. Our gene targeting studies show that the downstream  $V\gamma$  genes rearrange preferentially in the early fetal thymus because of their downstream location, independent of promoter or recombination signal sequences and unrelated to the extent of germline transcription. Remarkably, gene deletion studies show that the downstream  $V\gamma$  genes competitively inhibit upstream  $V\gamma$  rearrangements at the fetal stage. These data provide a mechanism for specialization of the fetal thymus for the production of T cells expressing specific  $V\gamma$  genes.

It has been estimated that TCR $\gamma\delta^+$  T cells exhibit substantially greater potential sequence diversity than TCR $\alpha\beta$  or primary Ig (1). Much of this diversity is caused by a great potential variability in CDR3 sequences in the TCR  $\delta$  chain, but there is also diversity in the number of V, J, and (in the case of  $\delta$ ) D gene segments. The  $\gamma$  locus consists of three functional  $J\gamma$ - $C\gamma$  genes and up to seven  $V\gamma$  genes, with four of the  $V\gamma$  genes clustered together upstream of  $J\gamma 1$ - $C\gamma 1$  (Fig. 1 B) (2–4).

Despite all of the potential diversity, the expressed diversity of  $\gamma\delta$  TCRs differs dramatically depending on the location of TCR $\gamma\delta^+$  T cells in the adult. Sessile populations of intraepithelial TCR $\gamma\delta^+$  T cells are found in the epidermis and the vaginal and tongue epithelium (5). The epidermal TCR $\gamma\delta^+$  T cells, called dendritic epidermal T cells (DETCs), all express  $V\gamma 3$  and  $V\delta 1$ , whereas TCR $\gamma\delta^+$  T cells in the vaginal and tongue epithelium all express  $V\gamma 4$  and  $V\delta 1$  (6). There is essentially no TCR $\gamma\delta$  diversity in these

locations, as all of the cells express the same  $V\gamma$  and  $V\delta$ , and the corresponding chains essentially lack N regions or other types of junctional diversity (7). Accordingly, TCR $\gamma\delta^+$  T cells in these locations have been called “invariant  $\gamma\delta$  T cells.”

TCR $\gamma\delta$  receptors in other locations show much more diversity. Many intraepithelial  $\gamma\delta$  T cells resident in the intestinal epithelium use  $V\gamma 5$  associated with various  $V\delta$  chains, but these chains, unlike the invariant intraepithelial cells, show much more junctional diversity (8). In secondary lymphoid organs,  $\gamma\delta$  T cells using  $V\gamma 2$ ,  $V\gamma 5$ ,  $V\gamma 1.1$ , and  $V\gamma 1.2$  are predominant, associated with numerous  $V\delta$  chains (2). Again, these chains show substantial junctional diversity. The striking differences between the repertoires of invariant  $\gamma\delta$  T cells and  $\gamma\delta$  T cells in the secondary lymphoid organs suggest different functions for these cells. Invariant  $\gamma\delta$  T cells such as DETCs are thought to recognize predictable self-ligands, which are up-regulated by cell stress such as that associated with wounding or tumorigenesis (9–11). Some of the diverse TCR $\gamma\delta^+$  T cells in secondary lymphoid organs also recognize self-ligands (12), but others may play a more conventional immunological role by recognizing variable antigens

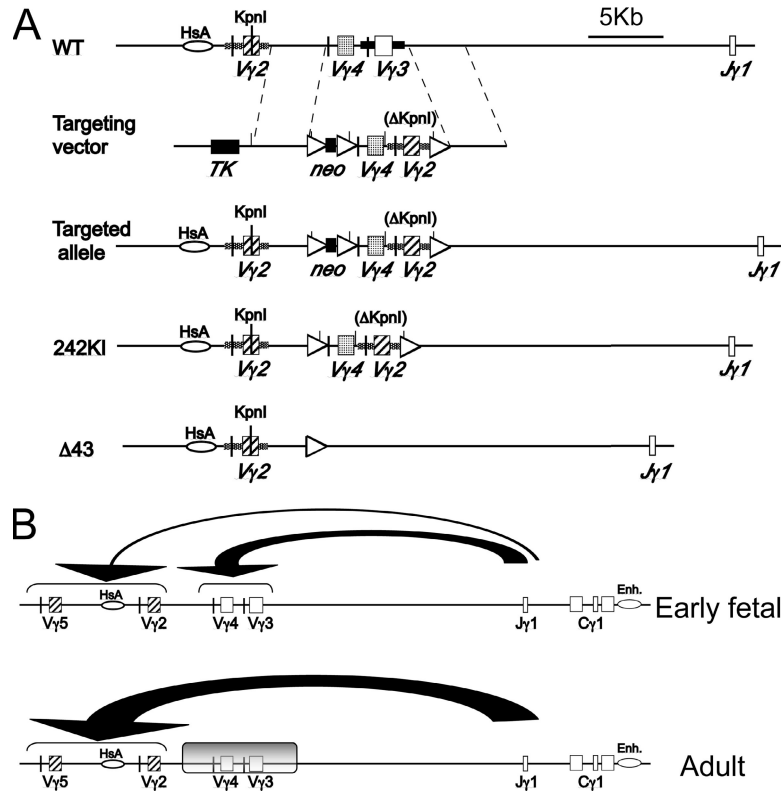
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Abbreviations used: DETC, dendritic epidermal T cell; E, embryonic day; ES, embryonic stem; KI, knock-in; RSS, recombination signal sequence(s); TdT, terminal deoxynucleotidyl transferase.

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**Figure 1. Gene targeting approach to insert a second downstream copy of the  $V\gamma 2$  gene and delete the downstream  $V\gamma 4$  and  $V\gamma 3$  genes.** (A) Arrangement of wild-type, targeted, 242KI, and  $\Delta 43$  alleles. Maps are drawn to scale. The targeted allele replaces the downstream  $V\gamma 4$ - $V\gamma 3$  pair with a  $V\gamma 4$ - $V\gamma 2$  pair flanked by loxP sites and a loxP-flanked *neo* cassette. Transfection of targeted ES cell clones with a Cre expression plasmid yielded clones in which only the *neo* cassette was deleted (242KI allele), or the entire segment contained  $V\gamma 4$ - $V\gamma 2$ -*neo* ( $\Delta 43$  allele). (B) Model of regulated  $V\gamma$  gene rearrangement during ontogeny. In the fetal period, all of the genes are in an open configuration, and the closer  $V\gamma 3$  and  $V\gamma 4$  genes enjoy an advantage in rearrangement because of their downstream location proximal to  $J\gamma 1$ . At the adult stage, repression of the downstream  $V\gamma 4$  and  $V\gamma 3$  genes through elements associated with the promoter segments overrides the downstream advantage resulting in preferential rearrangement of the upstream  $V\gamma 2$  and  $V\gamma 5$  gene segments.

associated with pathogens, consistent with the much greater receptor diversity observed in these populations.

A key question is how such different repertoires of TCR $\gamma\delta^+$  T cells can be produced during development of the immune system. A striking finding was that invariant  $V\gamma 3^+$  and  $V\gamma 4^+$  TCR $\gamma\delta^+$  T cells are only produced in the early fetal thymus (between embryonic days 13 and 17 [E13–17]), whereas the thymus switches to producing only the variable  $V\gamma 2^+$ ,  $V\gamma 5^+$ ,  $V\gamma 1.1^+$ , and  $V\gamma 1.2^+$  populations after E16 (13–15) (for review see reference 16). This specialization is determined by the stage of development of both precursor T cells and thymic stroma (5, 17). The switch has been shown to consist of both programmed events that determine the types of rearrangements that occur at different stages of ontogeny, and a cellular selection process that operates at least in the fetal period to select cells of defined specificity for export to the appropriate peripheral location, such as the skin (for review see reference 18).

Some of the relevant mechanisms are exemplified by the *Cy1* cluster, where the  $V\gamma 3$  and  $V\gamma 4$  genes expressed by invariant TCR $\gamma\delta^+$  T cells are located in the downstream position, closer to  $J\gamma 1$ , whereas the  $V\gamma 5$  and  $V\gamma 2$  genes expressed by many variable TCR $\gamma\delta^+$  T cells are located upstream (Fig.

1 A). The  $V\gamma$  gene rearrangement pattern changes in ontogeny so that the  $V\gamma 3$  and  $V\gamma 4$  genes rearrange preferentially in the early fetal period, whereas the upstream  $V\gamma 2$  and  $V\gamma 5$  genes rearrange preferentially in the adult stage (13). Studies with mutated TCR $\gamma$  transgenes or TCR $\delta$  knockout mice show that the stage-specific rearrangement pattern is independent of cellular selection processes (16, 19, 20). Rearrangements that occur in the fetal period usually lack N regions (as a result of the absence of terminal deoxynucleotidyl transferase [TdT]) and exhibit little or no junctional diversity (21, 22), whereas the adult-stage rearrangements contain abundant N regions and junctional diversity. On top of these programmed events in development, there is considerable evidence for a selection process that acts in the fetal thymus to select  $V\gamma 3^+$  cells that express appropriate V $\delta 1$  chains, and to equip these cells with homing receptors necessary for export from the thymus and emigration to the epidermis (23, 24).

Previous studies of V(D)J rearrangement suggest several possible mechanisms for differential V gene rearrangements. Early studies of  $V_H$  genes (25, 26), later extended to  $V\gamma$  and  $V\delta$  gene families (13, 27), showed that rearrangements of downstream V genes are often favored in the fetal period,

raising the possibility that the downstream location is inherently favorable for rearrangement at this stage. Later studies showed a correlation between germline transcription of rearranging gene segments and subsequent rearrangements (28, 29) (for review see reference 30). Studies with isolated nuclei showed that the local “accessibility” of rearranging gene segments to exogenously added RAG recombinase is also correlated with subsequent rearrangement (31). The relative roles of transcription versus simple accessibility have proven challenging to dissect, but a recent report provided direct evidence that germline transcription is causally related to rearrangement in the TCR $\alpha$  locus (32). Transcription, accessibility, and gene rearrangement have also been linked in some cases to local histone acetylation and/or lack of methylation of gene segments (33, 34). Finally, the specific sequence of a recombination signal sequence (RSS) can restrict its capacity to recombine with other RSS, despite compatibility with the 12/23 joining rule (35). Any or all of these mechanisms may work together or separately to impose the observed pattern of rearrangements.

In previous experimental analyses of the TCR $\gamma$  locus, we have focused on the upstream *V $\gamma$ 2* gene as a representative adult-stage gene, and the downstream *V $\gamma$ 3* gene as a representative fetal-stage gene. In adult stage thymocytes, the upstream *V $\gamma$ 2* gene rearranges  $\sim$ 25 times more frequently than the downstream *V $\gamma$ 3* gene (20, 36). We implicated the *V $\gamma$*  promoter sequences in this regulation by showing that the preference for *V $\gamma$ 2* over *V $\gamma$ 3* rearrangements in adult thymocytes, as well as the germline transcription pattern, was reversed when the promoter regions of the *V $\gamma$ 3* and *V $\gamma$ 2* genes were swapped in a transgenic backbone consisting of unrearranged *V $\gamma$ 2-4*, *J $\gamma$ 1*, and *C $\gamma$ 1* gene segments (20, 36). Other studies showed that poor rearrangement of *V $\gamma$ 3* in adult thymocytes correlates with low germline transcription and low levels of histone acetylation (29, 34) of the *V $\gamma$ 3* gene as compared with the *V $\gamma$ 2* gene. Furthermore, inhibitors of histone deacetylase were shown to enhance *V $\gamma$ 3* rearrangements in thymic organ cultures reconstituted with adult bone marrow stem cells (34). These data indicated that rearrangement of *V $\gamma$ 4/V $\gamma$ 3* genes is strongly repressed in the adult stage by a mechanism that depends at least in part on the V gene promoter segments.

Two lines of evidence suggest that the pattern of rearrangement is regulated by a distinct mechanism in the early fetal thymus. First, although the downstream *V $\gamma$ 3* gene rearranges approximately four times more frequently than the upstream *V $\gamma$ 2* gene in fetal thymocytes (20, 36), both genes were germline transcribed at a relatively high level and showed similarly high levels of histone acetylation (34, 36). Second, swapping the *V $\gamma$ 3* and *V $\gamma$ 2* promoter regions in a transgenic construct did not reverse the fetal pattern of rearrangement, as it did the adult pattern (20). In light of these and additional data with transgenic rearrangement constructs, we proposed that *V $\gamma$ 2* and *V $\gamma$ 3* genes are similarly accessible and transcriptionally active in fetal thymocytes, and that the preference for rearrangement of *V $\gamma$ 3* arises because of an inherent preference for rearrangement of the *V $\gamma$*  gene that lies closest to *J $\gamma$ 1*

(18, 36). In addition, we suggested that the preference is established at least in part because of a competition in which the rearrangement of J-proximal *V $\gamma$*  genes inhibits the rearrangement of more distal *V $\gamma$*  genes. In the adult stage, repression of the *V $\gamma$ 3* and *V $\gamma$ 4* genes is proposed to prevent rearrangement of these segments, thus removing the competitive barrier to *V $\gamma$ 2* rearrangement (Fig. 1 B). In this study, we have used a gene targeting approach to directly test two aspects of this proposal in the context of the endogenous TCR $\gamma$  locus. The results provide definitive support for the contentions that the downstream *V $\gamma$*  genes enjoy an inherent preference for rearrangement, and that rearrangement of the downstream *V $\gamma$*  genes competitively inhibits rearrangement of the upstream *V $\gamma$ 2* gene segment. Furthermore, both of these effects occur independently of changes in germline transcription, suggesting that the position of the *V $\gamma$*  gene by itself determines the rearrangement pattern in the fetal thymus.

## RESULTS

### Gene-targeted mice to test the role of gene location in programmed V gene rearrangement

To address the role of V gene location in V gene rearrangement in the endogenous TCR *C $\gamma$ 1* locus, we used gene targeting to create a locus with two *V $\gamma$ 2* genes, one in the normal upstream position and the second at the downstream position normally occupied by the *V $\gamma$ 3* gene (Fig. 1 A). This arrangement created the opportunity to examine the rearrangement of essentially identical *V $\gamma$*  genes located in distinct locations in the genome. To create this locus, an endogenous gene segment containing the downstream *V $\gamma$ 4-V $\gamma$ 3* gene pair was replaced by homologous recombination with an in vitro-assembled *V $\gamma$ 4-V $\gamma$ 2* pair flanked by loxP sites and a *neo* cassette, in which the 2.3-kb fragment containing *V $\gamma$ 2* included the *V $\gamma$ 2* promoter region, RSS, and flanking sequences (Fig. 1 A; and Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20071275/DC1>). The introduced *V $\gamma$ 2* copy contained a silent single bp substitution that destroyed a KpnI site in the middle of the *V $\gamma$ 2* gene, providing the basis for assays to distinguish this sequence from the wild-type upstream copy when analyzing genomic DNA or mRNA. The loxP sites flanking *V $\gamma$ 4-V $\gamma$ 2* provided a separate option to delete these *V $\gamma$*  genes in the germline, as described below. After deleting the *neo* cassette by transfection of embryonic stem (ES) cells with a *Cre* expression plasmid (Fig. 1 and Fig. S1), we achieved germline transmission of the knock-in (KI) allele, named 242KI. Homozygous 242KI/242KI mice and +/+ littermates were generated by intercrossing (Fig. S1). Because the ES cells were from the 129/SvJ strain, the founder mice were crossed to 129/J mice to minimize genetic diversity and segregation.

### Preferential rearrangement of the downstream (J-proximal) *V $\gamma$ 2* gene in the fetal thymus of 242KI/242KI mice

The extent of *V $\gamma$*  gene rearrangement in fetal (E14 or E15) or adult thymocytes was determined by semiquantitative PCR or by Southern blotting. The relative levels of rearrangement

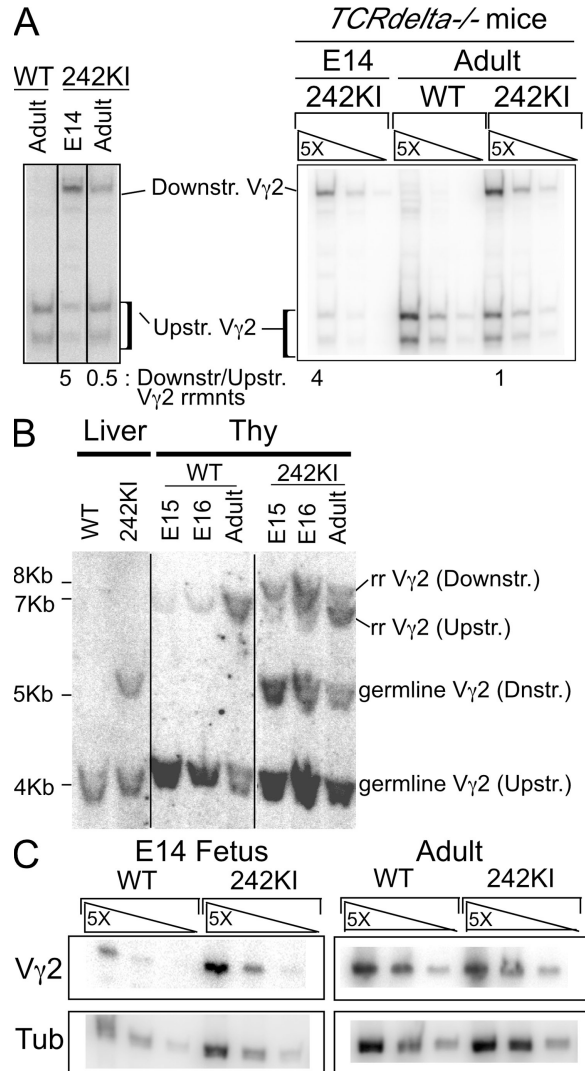
of the two  $V\gamma 2$  genes were determined by digesting the PCR products with KpnI (Fig. 2 A, left), or by performing Southern blots with DNA digested with restriction enzymes that allowed the two rearrangements to be distinguished by size (Fig. 2 B). In E14 fetal thymocytes, the downstream  $V\gamma 2$  gene segment rearranged four to five times more frequently than the upstream  $V\gamma 2$  gene. In E15 fetal thymocytes, the difference decreased to twofold (Fig. 2, A and B). Accordingly, the total level of  $V\gamma 2$  rearrangements (the sum of rearrangements of the upstream and downstream  $V\gamma 2$  genes) was elevated approximately fourfold in the 242KI E14 fetal thymocytes compared with wild-type fetal thymocytes (Fig. 2 C). The substituted  $V\gamma 2$  gene in the downstream position did not detectably alter the extent of  $V\gamma 4$  rearrangements (unpublished data).

It remained possible that these estimates of relative  $V\gamma$  gene rearrangement levels were distorted by cellular selection that influenced the abundance of cells with different rearrangements. To address this possibility, we crossed the 242KI mice to  $TCR\delta^{-/-}$  mice, thus preventing formation of a  $\gamma\delta$  TCR that could influence cellular selection. Analysis of 242KI/ $TCR\delta^{-/-}$  fetal thymocyte DNA by PCR showed that the downstream  $V\gamma 2$  rearrangements were favored over upstream  $V\gamma 2$  rearrangements to a similar extent as in 242KI/ $TCR\delta^{+/+}$  mice (Fig. 2 A, right).

The four- to fivefold preference for rearrangement of the downstream  $V\gamma 2$  gene in 242KI/242KI mice is comparable to the approximately fourfold preference for rearrangement of the downstream  $V\gamma 3$  gene observed in wild-type fetal thymocytes (20, 36). Therefore, the location of the  $V\gamma 2$  gene segment in the V gene array determines its preference for rearrangement in the fetal period independently of the RSS or the upstream promoter regions, which were identical in the two  $V\gamma 2$  genes under comparison.

#### Deletion of $V\gamma 4$ and $V\gamma 3$ results in increased rearrangement of $V\gamma 2$ at the fetal stage

Preferential rearrangement of the J-proximal  $V\gamma$  gene at the fetal stage might reflect a competition between  $V\gamma$  genes for rearrangement to  $J\gamma 1$ , in which the downstream  $V\gamma$  gene holds an advantage. If this were the case, deletion of the downstream  $V\gamma$  genes should result in an increased frequency of rearrangements of the upstream  $V\gamma$  gene. Deletion of the downstream  $V\gamma$  genes was possible because the  $V\gamma 4$ - $V\gamma 2$  gene pair in the targeted locus was flanked by loxP sites. Among the *Cre*-transduced ES cell colonies, we identified some in which this entire segment, as well as the *neo* cassette, were deleted by *Cre*-mediated recombination (Fig. 1 and Fig. S1). The deletion reduced the distance between  $V\gamma 2$  and  $J\gamma 1$  by only a modest 20% compared with the wild-type locus. The knockout allele was named  $\Delta 43$  to signify the fact that this targeted allele corresponds to a deletion of the  $V\gamma 4$  and  $V\gamma 3$  genes relative to the wild-type locus. After generating chimeras with the recombinant ES cells and achieving germline transmission of the  $\Delta 43$  allele by crossing to 129/J mice, homozygous  $\Delta 43/\Delta 43$  mice were generated by intercrossing.



**Figure 2.  $V\gamma 2$  gene rearrangements in 242KI/242KI and wild-type (+/+) thymocytes.** (A) PCR analysis of rearrangements of upstream versus downstream  $V\gamma 2$  genes in E14 fetal and adult thymocytes. Radiolabeled PCR products were amplified from thymocyte DNA with primers L2 and J1, digested with KpnI, and subjected to electrophoresis and PhosphorImager analysis (as described in Materials and methods). Parallel PCR analysis of wild-type genomic DNA was included to demonstrate complete KpnI digestion of PCR products of the wild-type (upstream)  $V\gamma 2$  gene. (left) Thymocytes from  $TCR\delta^{+/+}$  mice. (right) Thymocytes from  $TCR\delta^{-/-}$  mice, to examine rearrangement under conditions in which TCR $\gamma\delta$  receptors cannot be expressed. The 242KI mice in this experiment only had been backcrossed to C57BL/6J. (B) Southern analysis of  $V\gamma 2$  gene rearrangements in BamHI/NcoI-digested adult thymocyte and liver DNA hybridized with a  $V\gamma 2$  probe. Bands corresponding to rearranged (rr) and germline upstream (endogenous) and downstream (knocked-in)  $V\gamma 2$  genes are indicated. The ratio of downstream/upstream  $V\gamma 2$  rearrangements was 2.1-fold in E15 fetal thymocyte DNA, compared with 0.4-fold in adult thymocytes. Vertical black lines in A and B indicate lanes deleted in construction of the figure. (C) Semiquantitative PCR analysis of total  $V\gamma 2$  gene rearrangements (the sum of upstream and downstream [if present]  $V\gamma 2$  rearrangements) or control tubulin sequences in E14 fetal or adult thymocyte samples.  $V\gamma 2$  was rearranged fourfold more often in 242KI than in wild-type E14 fetal thymocytes, but there was no difference in adult double-negative thymocytes.

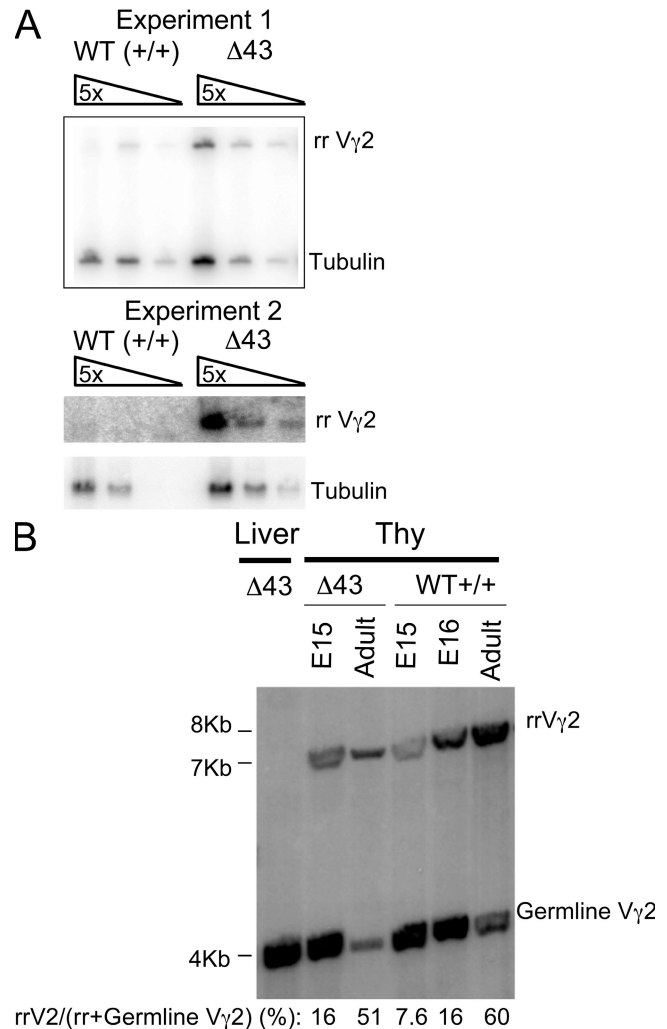


The amount of *Vγ2* gene rearrangement to *Jγ1* in  $\Delta43/\Delta43$  mice was compared with that in wild-type (+/+) littermates on the 129 background. As shown by semiquantitative PCR, the deletion of *Vγ3* and *Vγ4* resulted in an approximately fivefold increase in *Vγ2* rearrangement in E14 fetal thymocytes (based on three experiments; Fig. 3 A and not depicted). Thus, deletion of *Vγ3* and *Vγ4* resulted in increased levels of *Vγ2* rearrangement in E14 fetal thymocytes to about the level of *Vγ3* rearrangement observed in wild-type mice. Comparable PCR analysis (not depicted) and genomic Southern blotting with a *Vγ2* probe (Fig. 3 B and not depicted) revealed that *Vγ2* rearrangements were still elevated in  $\Delta43/\Delta43$  mice on E15, though to a lesser extent. A separate examination of rearrangements of *Vγ5*, which is also upstream of *Vγ3/Vγ4*, showed an approximately threefold increase in  $\Delta43/\Delta43$  mice compared with wild-type mice in E14 fetal thymocytes (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20071275/DC1>). These data indicated that rearrangements of the proximal *Vγ3* and *Vγ4* genes in the wild-type locus normally competitively inhibit rearrangements of the more distal *Vγ2* and *Vγ5* genes.

#### Location-dependent rearrangement patterns in the fetal thymus are not associated with changes in germline transcription

Because germline transcription has been causally associated with V(D)J rearrangements, we assessed whether the rearrangement patterns we observed correlated with the abundance of germline transcripts. To quantify germline transcripts, dilutions of cDNA generated by RT with random hexamer primers were PCR amplified with an upstream primer corresponding to the *Vγ2* coding region and a downstream primer corresponding to a sequence just 3' of the *Vγ2* gene RSS. No products were observed when RT was omitted from the reaction, confirming that the products corresponded to transcripts as opposed to contaminating genomic DNA (Fig. 4 A). For analysis of germline transcripts in thymocytes from 242KI/242KI mice, the PCR products were digested with KpnI to discriminate whether they originated from the wild-type upstream gene (cleaved) or the knocked-in downstream gene (uncleaved). Based on this analysis, germline transcripts of the downstream *Vγ2* gene were, if anything, slightly less abundant than upstream *Vγ2* germline transcripts in E14 fetal thymocytes (ratio = 0.83), despite the much increased level of rearrangement of the downstream gene at this time point.

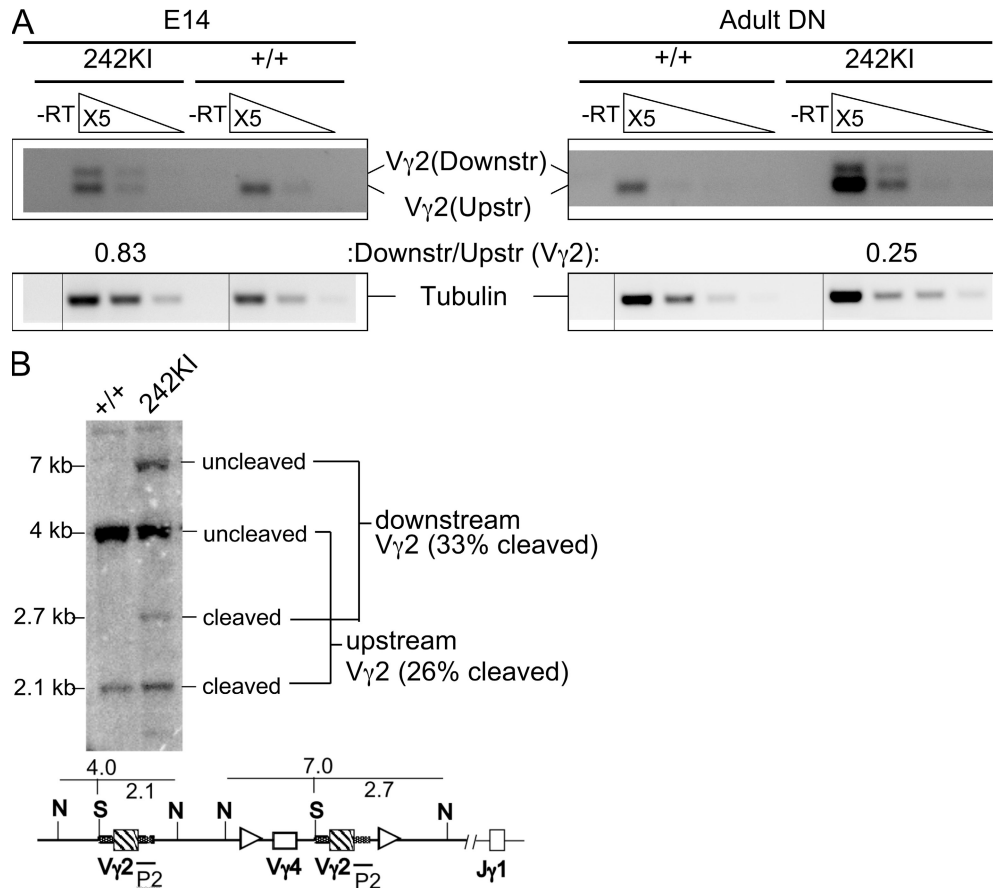
Two caveats should be noted in interpreting the germline transcription data. First, a correction should be applied to these data, because preferential rearrangement of the downstream gene copy is predicted to decrease the availability of unrearranged downstream gene copies, compared with upstream copies, for transcription. However, it is likely that this correction is negligible in light of previous data showing that <1% of total gene copies are rearranged in E14 thymocytes (20, 36). The second caveat is that it is conceivable that the altered nucleotide sequence of the knocked-in



**Figure 3. *Vγ2* gene rearrangements in  $\Delta43/\Delta43$  mice.** (A) Two experiments examining *Vγ2* rearrangements in E14 fetal thymocytes of wild-type and  $\Delta43/\Delta43$  mice by semiquantitative PCR. The PCR for tubulin was used as a loading control. (B) Southern blot analysis of *Vγ2* gene rearrangements in BamHI/NcoI-digested adult thymocyte or liver genomic DNA hybridized with a *Vγ2* probe. Bands corresponding to germline and rearranged (rr) *Vγ2* genes are indicated. The percentage of rearranged/(rearranged + unrearranged) *Vγ2* alleles in each lane is presented at the bottom.

downstream copy of *Vγ2* resulted in altered stability or processing of the corresponding germline transcripts, resulting in an underestimate of the level of germline transcription. We think it unlikely, however, that the single bp change introduced near the middle of the *Vγ2* exon would cause such a substantial change in transcript stability or processing. Based on these considerations, the data support the conclusion that the higher level of rearrangement of the 3' *Vγ2* gene in fetal thymocytes occurs in the absence of increases in germline transcription.

We also used a restriction enzyme accessibility assay to determine whether the two copies of the *Vγ2* gene were differentially accessible in the nuclei of E15 fetal thymocytes



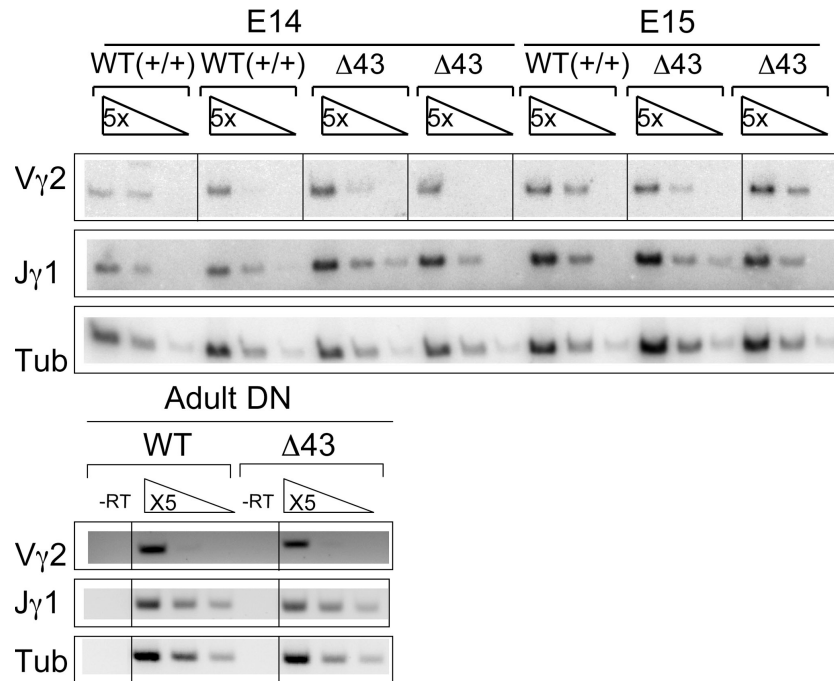
**Figure 4.** *V* $\gamma$ 2 gene germline transcription and restriction enzyme accessibility in 242KI mice. (A) Comparison of germline transcripts of the upstream versus downstream *V* $\gamma$ 2 gene in E14 fetal thymocytes and adult double-negative (DN) thymocytes from 242KI/242KI mice by semiquantitative RT-PCR (see Materials and methods). The PCR products were digested with KpnI before electrophoresis and PhosphorImager analysis to distinguish their origin from the downstream (uncleaved) versus upstream (cleaved; note that the smaller fragment has run off the gel) *V* $\gamma$ 2 genes (see Materials and methods). Semiquantitative RT-PCR for tubulin was used as a loading control. The ratios of downstream/upstream *V* $\gamma$ 2 rearrangements are depicted below the panels (means of two assays). Vertical black lines indicate lanes deleted in construction of the figure. –RT, sample without RT. (B) Accessibility to *Scal* cleavage of *Scal* restriction enzyme sites located upstream of both *V* $\gamma$ 2 gene copies in the nuclei of 242KI E15 fetal thymocytes. The bands corresponding to upstream and downstream *V* $\gamma$ 2 genes that were accessible (cleaved) versus inaccessible (uncleaved) to *Scal* are indicated on the right, along with the percentage of cleavage.

from 242KI/242KI mice. A *Scal* restriction enzyme site  $\sim$ 380 bp upstream of the *V* $\gamma$ 2 ATG codon was nearly equally accessible to restriction enzyme in the two copies of *V* $\gamma$ 2 in 242KI/242KI mice in the nuclei of E15 fetal thymocytes (Fig. 4 B). Therefore, the different levels of rearrangement did not correlate with the accessibility of this site to restriction enzyme.

To assess whether the increased rearrangement of *V* $\gamma$ 2 in fetal thymocytes from  $\Delta$ 43/ $\Delta$ 43 mice could be attributed to greater germline transcription, we quantified *V* $\gamma$ 2 germline transcripts in  $\Delta$ 43/ $\Delta$ 43 versus +/+ E14 and E15 fetal thymocytes. In two experiments, fetal thymocytes from the two strains contained essentially identical levels of *V* $\gamma$ 2 germline transcripts (Fig. 5 and not depicted). Thus, the substantial increase in *V* $\gamma$ 2 rearrangements that accompanied deletion of *V* $\gamma$ 4 and *V* $\gamma$ 3 is not accompanied by a corresponding increase in *V* $\gamma$ 2 germline transcription.

#### Influence of V gene location on the rearrangement and accessibility of *V* $\gamma$ genes at the adult stage

In adult-stage wild-type thymocytes, *V* $\gamma$ 3 and *V* $\gamma$ 4 rearrangements are normally strongly depressed compared with the fetal stage. We therefore tested whether replacing *V* $\gamma$ 3 with *V* $\gamma$ 2 in 242KI/242KI mice prevents the repression of downstream *V* $\gamma$  rearrangements in adult thymocytes. The abundance of upstream *V* $\gamma$ 2 rearrangements was higher in adult than fetal thymocytes of 242KI mice, just as it was in wild-type mice (Fig. 2 B). However, in contrast to the situation in fetal thymocytes, the majority ( $\sim$ 70%) of *V* $\gamma$ 2 rearrangements in adult 242KI/242KI thymocytes involved the upstream *V* $\gamma$ 2 gene (Fig. 2, A and B), representing an  $\sim$ 10-fold decrease in downstream *V* $\gamma$ 2 rearrangements relative to upstream *V* $\gamma$ 2 rearrangements between the fetal and adult stages. Although there was a shift toward upstream *V* $\gamma$ 2 rearrangements in adult 242KI thymocytes, the overall preference for rearranging the



**Figure 5. Germline transcription of  $V\gamma 2$  and  $J\gamma 1$  genes in  $\Delta 43/\Delta 43$  versus wild-type fetal thymocytes.** E14 and E15 fetal, and adult  $CD4^-CD8^-$  thymocyte RNA samples were reverse transcribed with random hexamer primers and subjected to semiquantitative PCR analysis with  $V\gamma 2$  or  $J\gamma 1$  primer sets to detect germline transcripts (see Materials and methods). Two replicate samples were run in parallel in some cases, as indicated. The ratios of  $V\gamma 2$  germline transcripts in wild-type mice versus  $\Delta 43/\Delta 43$  mice, calculated from PhosphorImager data and normalized with the PCR for  $\beta$ -tubulin transcripts, were 0.9 for E14 fetal thymocytes, 1 for E15 fetal thymocytes, and 1 for adult thymocytes (mean of at least two samples for each). The ratios of  $J\gamma 1$  germline transcripts in wild-type versus  $\Delta 43/\Delta 43$  mice were 0.7 for E14 fetal thymocytes, 1 for E15 fetal thymocytes, and 1.1 for adult thymocytes (mean of at least two samples for each). Samples lacking RT (–RT), run in parallel, yielded no PCR products, as shown for adult thymocytes (bottom). Vertical black lines indicate lanes deleted in construction of the figure.

upstream gene was smaller in 242KI mice (approximately 2-fold) than it was in adult wild-type thymocytes (which exhibit a 25-fold preference for  $V\gamma 2$  over  $V\gamma 3$  rearrangements).

We also determined whether the decreased proportion of downstream  $V\gamma 2$  rearrangements at the adult stage was paralleled by a decrease in the proportion of germline transcripts emanating from the downstream gene. The analysis showed a reduction of approximately threefold in the proportion of germline transcripts emanating from the downstream gene (Fig. 4 A). These data must be adjusted based on the relative number of copies of each unrearranged  $V\gamma 2$  gene in the population being examined. A separate PhosphorImager analysis (see Materials and methods) of three Southern blots, examining band intensity in thymocytes as compared with liver DNA, showed that the ratio (downstream/upstream) of unrearranged  $V\gamma 2$  genes in adult thymocytes was  $\sim 0.74$  (unpublished data). Hence, the abundance of  $V\gamma 2$  germline transcripts emanating from the downstream  $V\gamma 2$  gene was reduced by  $\sim 2.2$ -fold after normalization for the number of gene templates in the population. The decreased proportion of rearrangements and germline transcripts of the downstream  $V\gamma 2$  gene segment in adult thymocytes suggests that sequences in the vicinity of the downstream gene repress both germline transcription and rearrangement at the adult stage. Because the downstream  $V\gamma 2$  gene segment is identical to the upstream segment, the relevant

repressive sequences are presumably not in the downstream  $V\gamma 2$  gene segment itself, but more likely in neighboring sequences. Hence, these data are consistent with the existence of sequences in the downstream region in addition to those previously attributed to the  $V\gamma 3$  promoter (which is absent from the 242KI chromosome) (20), which locally repress rearrangements in the adult thymus.

In adult thymocytes, rearrangements of the upstream  $V\gamma 2$  gene were similarly abundant in wild-type mice as in either 242KI/242KI (Fig. 2 B) or  $\Delta 43/\Delta 43$  (Fig. 3 B) mice. Furthermore, in adult thymocytes,  $V\gamma 2$  germline transcripts were similarly abundant in wild-type, 242KI/242KI (Fig. 4 A, legend), and  $\Delta 43/\Delta 43$  (Fig. 5) thymocytes. Hence, the alterations at the downstream position in these two chromosomes did not alter the efficiency of rearrangements or germline transcription at the upstream position in adult thymocytes.

## DISCUSSION

Previous studies (20, 36) demonstrated that the downstream  $V\gamma 3$  gene is preferentially rearranged in the early fetal thymus compared with  $V\gamma 2$  by a factor of approximately fourfold. Other studies show that the fraction of productive rearrangements is inherently lower for  $V\gamma 2$  than for rearrangements of other V genes, because  $V\gamma 2$  uniquely contains an in-frame stop codon near its 3' end, which must be removed (presumably by

endonuclease action) during the recombination process (16). The relative paucity of  $V\gamma 2$  rearrangements at the fetal stage, coupled with the rarity of productive  $V\gamma 2$  rearrangements, are critical in ensuring that nearly all TCR $\gamma\delta^+$  fetal thymocytes express  $V\gamma 3$  (or  $V\gamma 4$ ) and not  $V\gamma 2$ . The absence of TdT at the fetal stage also plays a role in the process of  $V\gamma 3$  gene rearrangement. Without TdT and the N nucleotides it adds, the frequency of rearrangements that occur at the site of a dinucleotide homology shared by the  $V\gamma 3$  and  $J\gamma 1$  gene segments is increased, thus ensuring that most productive rearrangements carry precisely the same (canonical)  $V\gamma$ - $J\gamma$  junctional sequence (22). These mechanisms, coupled with a selection process for  $V\gamma 3^+$  T cells that operates in the fetal thymus (24), conspire to convert the early fetal thymus into an organ specialized in part for the production of  $V\gamma 3^+$  DETCs.

Our data using gene-targeted chromosomes address a key part of this coordinated process by probing the mechanisms that lead to preferential  $V\gamma 3$  gene rearrangement in the fetal thymus. The results have implications for other Ig/TCR genes as well, because preferential rearrangement of J-proximal V genes is also observed in early stage development of B cells and T cells in the case of the IgH and TCR $\delta$  gene families. The results of this study provide compelling *in vivo* evidence that the location of a  $V\gamma$  gene is a critical determinant of its rearrangement pattern in the fetal thymus and provide direct evidence that the downstream gene exerts competitive inhibition of upstream V gene rearrangement at the fetal stage.

Previous studies provided evidence that at the fetal stage, the unrearranged  $V\gamma 2$  and  $V\gamma 3$  gene segments are acetylated on histone residues to a similar extent, and are germline-transcribed similarly as well (34, 36). These similarities suggested that neither accessibility nor transcriptional activity of the genes was a likely explanation for the preferential rearrangement of the downstream  $V\gamma$  genes, raising the possibility that gene location by itself plays a role in rearrangement at the fetal stage. This interpretation was further suggested by our previous study performed with transgenes consisting of unrearranged  $V\gamma/J\gamma/C\gamma$  genes, in which the positions of the  $V\gamma 3$  and  $V\gamma 2$  gene segments were swapped within the transgene (36). The present study, however, is a definitive analysis, as it was performed by targeting the endogenous locus, and is therefore free of certain artifacts that can confound interpretation of transgenic mice, such as the existence of multiple tandem copies of the transgene that create the opportunity for complex rearrangements to take place and that may override natural regulatory processes, as well as the potential absence of flanking regulatory sequences that may influence the rearrangement process. Equally important, the current study is superior as it examines two copies of the same complete  $V\gamma$  gene segment ( $V\gamma 2$ ) located at the upstream and downstream positions. Hence, differences in rearrangement cannot be attributed to differences in the RSS or promoters of the genes, or other features associated with the coding and immediately flanking DNA, all of which were included in the inserted downstream  $V\gamma 2$  copy.

The results showed that the downstream  $V\gamma$  gene enjoyed an approximately fivefold advantage in rearrangement frequency in the early fetal thymus. The upstream and downstream  $V\gamma$  genes were germline transcribed to a similar extent, suggesting that the advantage did not result from greater accessibility or transcription-related enhancement of rearrangement. We propose, instead, that the advantage of the downstream  $V\gamma$  gene arises from a greater likelihood of encountering the  $J\gamma 1$  RSS, either because of its closer proximity or because of a processive aspect to the rearrangement process that favors the first encountered  $V\gamma$  gene. We cannot rule out the possibility that the advantage arises from closer proximity of the downstream gene to a downstream regulatory element. However, we previously showed that rearrangement is not significantly altered in the fetal thymus of mice with targeted deletions of either or both of the two enhancer-like elements in the locus, including the downstream enhancer  $E_{3/C\gamma 1}$  (37).

Based on these findings, we hypothesized that the fetal pattern of rearrangement is determined by a competition between the downstream and upstream  $V\gamma$  gene segments, with an advantage accruing to the downstream  $V\gamma$  gene owing to its closer proximity to  $J\gamma 1$  (Fig. 1 B). The design of our gene-targeted allele enabled us to test for the first time a key prediction of this hypothesis by determining whether deletion of the intervening  $V\gamma 4$  and  $V\gamma 3$  genes altered the putative competition so as to promote rearrangement of the upstream  $V\gamma 2$  gene. The results demonstrated that deletion of  $V\gamma 4$  and  $V\gamma 3$  resulted in enhanced rearrangements of the upstream  $V\gamma 2$  gene segment in the fetal thymus to an extent commensurate with the amount of rearrangement that normally occurs at the downstream  $V\gamma$  gene. These findings provide direct support for the notion that  $V\gamma$  genes are normally in competition in the fetal stage, and that J-proximal rearrangements occur at the expense of J-distal rearrangements. The small reduction in the length of the segment of DNA connecting  $V\gamma 2$  and  $J\gamma 1$  resulting from the deletion,  $\sim 20\%$ , appears unlikely to account for an approximately fivefold increase in rearrangement in E14 fetal thymocytes.

As discussed in the Introduction, the pattern of rearrangement in the adult thymus is imposed by a distinct mechanism that is regulated by  $V\gamma$  promoter-specific sequences and leads to repression of  $V\gamma 3$  and  $V\gamma 4$  rearrangements that is dependent on local histone deacetylation. Considering all these observations, we proposed that the  $V\gamma 3$  promoter contains sequences that locally repress  $V\gamma 3$  and  $V\gamma 4$  rearrangements in the adult, abolishing competition from downstream genes and enhancing upstream  $V\gamma$  gene rearrangements (18). The notion that  $V\gamma 3$ -associated sequences repress rearrangement in the adult thymus predicts that replacing  $V\gamma 3$  and its promoter with corresponding  $V\gamma 2$  sequences, as in the 242KI mice, should result in less effective silencing of downstream rearrangements in adult thymocytes. The data are consistent with this interpretation, because the bias in favor of rearrangement of the upstream  $V\gamma 2$  gene in adult 242KI/242KI mice (2-fold) was small in comparison to the bias in favor of  $V\gamma 2$  over  $V\gamma 3$  rearrangements in adult wild-type mice (25–50-fold). On the other hand, although



the overall level of rearrangement increased substantially between the fetal and adult stages, presumably because of enhanced overall activity of the locus, the relative abundance of downstream  $V\gamma 2$  rearrangements in adult thymocytes of 242KI/242KI mice declined by  $\sim 10$ -fold compared with the situation in fetal thymocytes. A plausible explanation for these data is that the 242KI allele retains residual repressive sequences active at the adult stage. One possibility is that the  $V\gamma 4$  gene, which remains in the 242KI allele, contains these putative residual repressive sequences.

The results reported in this paper support a significant role of gene position and V gene competition in controlling the abundance of specific V gene rearrangements. These features must therefore be considered in conjunction with transcriptional activity, accessibility, differences in RSS sequences, and other factors as important regulators of V(D)J recombination. All of these factors may be exploited in the evolution of mechanisms to create various specific repertoires in the adaptive immune system. This is illustrated by the dynamic behavior of the TCR $\gamma$  locus during development, in which the positional influence, gene competition and other factors work in tandem to enable the early fetal thymus to specialize in the production of invariant TCR $\gamma\delta^+$  T cells destined for epithelial locations, whereas transcriptional repression and/or loss of accessibility enable the adult thymus to switch its efforts toward the production of  $V\gamma 2^+$  and other TCR $\gamma\delta^+$  subsets found abundantly in secondary lymphoid organs in adult mice.

## MATERIALS AND METHODS

**Generation of 242KI and  $\Delta 43$  knockout mice.** A single gene targeting event was used to generate 242KI and  $\Delta 43$  knockout mice. The targeting vector (Fig. 1 A and Fig. S1) was based on the pKSTKNeoLoxP plasmid (a gift from R. Rickert, Burnham Institute for Medical Research, La Jolla, CA). It included a thymidine kinase cassette at the 5' end, a 5' homology region corresponding to a 3.2-kb *SnaBI*-*BglII* fragment upstream of the  $V\gamma 4$  gene, and a 3' homology region corresponding to a 3.2-kb *EcoRI*-*BamHI* fragment downstream of the  $V\gamma 3$  gene. Between the arms, we inserted a *PGK-neo* cassette and a  $V\gamma 4$ - $V\gamma 2$  gene pair comprising a 1.7-kb *BglII*-*HindIII*  $V\gamma 4$  fragment ligated to a 2.5-kb *SpeI*-*EcoRI*  $V\gamma 2$  fragment. The latter fragment was the same fragment used in an earlier transgenic study (20, 36), and it included the  $V\gamma 2$  promoter, leader exon, intron, coding segment, RSS, and 3' flanking DNA. In the present study, the inserted  $V\gamma 2$  fragment contained a silent 1-bp mutation that destroyed the unique *KpnI* site located in the coding region of this gene (gggtacc $\rightarrow$ gggtatc) to enable discrimination from the upstream wild-type gene. The *PGK-neo* cassette was flanked by loxP sites, and an additional loxP site was located downstream of  $V\gamma 2$  at the 3' end of the inserted segments (Fig. 1 A). The locations of the three loxP sites enabled us to delete the *PGK-neo* cassette or the entire inserted sequence, depending on whether the 5' loxP site recombined with the central loxP site or the 3' loxP site.

The targeting construct was transfected into J1 ES cells of the 129/SvJae strain (38), followed by selection with G418 and ganciclovir. Targeted ES cell clones were identified by Southern blotting with a 5' flanking probe (Fig. S1 A). Correct targeting at the 3' end was confirmed by PCR (Fig. S1 B). The targeted ES cell clones were transiently transfected with a Cre-expressing plasmid (39) and screened by Southern blotting for clones that had deleted only the *PGK-neo* cassette (242KI), or had deleted both the *PGK-neo* cassette and the  $V\gamma 4$ - $V\gamma 2$  fragment ( $\Delta 43$ ; Fig. S1 D). The recombinant ES cell clones were separately injected into C57BL/6 blastocysts, and chimeric mice were crossed with 129/J and C57BL/6J mice. Heterozygous pups from the 129 cross were intercrossed to generate homozygous 242KI/242KI or  $\Delta 43/\Delta 43$  mice on the 129 background. Heterozygous pups from the C57BL/6J

cross were serially backcrossed to C57BL/6J mice six times before intercrossing. Blotting genomic DNA with  $V\gamma 3$ ,  $V\gamma 2$ , and other probes confirmed germline transmission of the targeted alleles (Fig. S1 E and not depicted). Animal studies were approved and overseen by the University of California, Berkeley Animal Care and Use Committee.

**Cell, DNA, and RNA preparations.** Total and CD4 $^-$ CD8 $^-$  (double-negative) thymocytes were prepared as previously described (37). DNA and total cellular RNA were prepared as previously described (20).

**Primers.** Primers L2, J1, PSV $\gamma 2$ , VG2I, V2-3'a, 5'J $\gamma 1$  5'-2', C $\gamma 1$  3'-3, 5' tubulin, and 3' tubulin have been previously described (18, 20, 29, 34, 36). Primer PSV $\gamma 2'$  is as follows: tcgaagccttaggagtgaacaatacac.

**Semiquantitative PCR and RT-PCR analysis.** The semiquantitative PCR assays were performed essentially as previously described (20). In brief, thymic genomic DNA was subjected to PCR in the presence of  $\alpha$ -[ $^{32}$ P]deoxycytidine triphosphate (dCTP) with the primer set L2/J1 to amplify  $V\gamma 2$  gene rearrangements. To distinguish whether the radiolabeled PCR products originate from rearrangements of the endogenous upstream  $V\gamma 2$  gene versus the knocked-in downstream  $V\gamma 2$  gene in 242KI mice, they were digested with *KpnI* before electrophoresis on a polyacrylamide gel and analysis (PhosphorImager; Molecular Dynamics).

Semiquantitative RT-PCR performed as previously described (18, 36), with minor modifications, was used to quantify  $V\gamma 2$  or J $\gamma 1$  germline transcripts. RNA samples from E14 fetal thymocytes or adult CD4 $^-$ CD8 $^-$  thymocytes were treated with DNase to eliminate any genomic DNA contamination and reverse transcribed with Superscript II RNase H $^-$  RT using random hexamer primers. The RT products were serially diluted (by the factors indicated in the figures) and subjected to PCR in the presence of  $\alpha$ -[ $^{32}$ P]dCTP with primer sets PSV $\gamma 2$ /V2-3'a (for analysis of  $V\gamma 2$  transcripts in 242KI mice), PSV $\gamma 2'$ /V $\gamma 2$ -3'a (for analysis of  $V\gamma 2$  transcripts in  $\Delta 43$  mice), or 5'J $\gamma 1$  5'-2'/C $\gamma 1$  3'-3 (for J $\gamma 1$ ). In the case of samples from 242KI mice, the RT-PCR products (307 bp) were digested with *KpnI* to distinguish the origin of the products. RT-PCR products corresponding to the mutated 3'  $V\gamma 2$  copy cannot be cleaved by *KpnI*, whereas products corresponding to the endogenous 5'  $V\gamma 2$  gene are cleaved into smaller fragments (274 + 33 bp). Parallel semiquantitative RT-PCRs of  $\beta$ -tubulin transcripts were used to normalize the samples. In all cases, RNA samples without RT were subjected to PCR in parallel to assure that there was no genomic DNA contamination.

**Southern blot analysis of  $V\gamma 2$  gene rearrangements.** Thymic genomic DNA samples were digested with *NcoI*/*BamHI* and analyzed by Southern blotting using a  $V\gamma 2$ -specific probe, as previously described (20).

**Restriction enzyme accessibility assay.** The assay was performed to assess restriction enzyme accessibility of the two copies of  $V\gamma 2$  genes in 242KI mice. In brief, nuclei were prepared from E15 fetal thymocytes and digested with *ScaI* for 1 h. After the *ScaI* digestion, DNA was prepared from the nuclei and digested with *NcoI*. The DNA was analyzed by Southern blotting using a  $V\gamma 2$  probe.

**Online supplemental material.** Fig. S1 depicts and confirms the gene targeting procedure used to generate 242KI and  $\Delta 43$  mice. Fig. S2 shows changes in the rearrangement of  $V\gamma 5$  in  $\Delta 43$  mice. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20071275/DC1>.

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