# The Role of Recombination Activating Gene (*RAG*) Reinduction in Thymocyte Development In Vivo✪

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## Abstract

Assembly of T cell receptor (TCR)α/β genes by variable/diversity/joining (V[D]J) rearrangement is an ordered process beginning with recombination activating gene (*RAG*) expression and TCRβ recombination in $CD4^-CD8^-CD25^+$ thymocytes. In these cells, TCRβ expression leads to clonal expansion, *RAG* downregulation, and TCRβ allelic exclusion. At the subsequent $CD4^+CD8^+$ stage, *RAG* expression is reinduced and V(D)J recombination is initiated at the TCRα locus. This second wave of *RAG* expression is terminated upon expression of a positively selected α/β TCR. To examine the physiologic role of the second wave of *RAG* expression, we analyzed mice that cannot reinduce *RAG* expression in $CD4^+CD8^+$ T cells because the transgenic locus that directs *RAG1* and *RAG2* expression in these mice is missing a distal regulatory element essential for reinduction. In the absence of *RAG* reinduction we find normal numbers of $CD4^+CD8^+$ cells but a 50–70% reduction in the number of mature $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes. TCRα rearrangement is restricted to the 5′ end of the Jα cluster and there is little apparent secondary TCRα recombination. Comparison of the TCRα genes expressed in wild-type or mutant mice shows that 65% of all α/β T cells carry receptors that are normally assembled by secondary TCRα rearrangement. We conclude that *RAG* reinduction in $CD4^+CD8^+$ thymocytes is not required for initial TCRα recombination but is essential for secondary TCRα recombination and that the majority of TCRα chains expressed in mature T cells are products of secondary recombination.

Key words: T cell receptor α chain • gene rearrangement • regulation of gene expression • T cell receptor editing • recombination activating gene

## Introduction

During lymphocyte development immunoglobulin and TCR genes are assembled from germline V, D, and J gene segments by a site-specific recombination reaction (1). The V(D)J recombination reaction is mediated by the products of the lymphocyte specific recombination activating genes *RAG1** and *RAG2* which recognize and cleave recombination signal sequences located adjacent to the coding V, D, and J segments (2–5). T and B lymphocyte development requires V(D)J recombination; in the absence of RAG1 and RAG2 (6, 7) or factors that repair the double strand DNA breaks created during V(D)J recombination there is a complete block in the early stages of B and T cell development (8–15).

In thymocytes, V(D)J recombination is initiated at the TCRβ locus in $CD4^-CD8^-$ double negative (DN) T cells (16). Once a TCRβ chain is expressed it combines with pre-Tα and CD3 components to produce the pre-TCR complex (for reviews, see references 17 and 18). Pre-TCR expression downregulates *RAG* expression and induces T cells to mature to the $CD4^+CD8^+$ double positive (DP) stage. Upon entering the DP stage there is a second wave of *RAG* expression and V(D)J recombination (19–21). Regulation of *RAG* expression in developing thymocytes

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**Abbreviations used in this paper:* APC, allophycocyanin; BAC, bacterial artificial chromosome; DN, double negative; DP, double positive; NBS, Nijmegen breakage syndrome; SP, single positive; RAG, recombination activating gene; RT, reverse transcription; TEA, T early α promoter; YAC, yeast artificial chromosome.

has been studied by two groups (22, 23). Transgenic experiments with large bacterial artificial chromosomes (BACs) that carry fluorescent protein indicator genes in place of the *RAG* genes, showed that a cis element 35–70 kb 5′ of *RAG2* is required for the second wave of *RAG* expression (22). However, *RAG2*$^{-/-}$ blastocyst reconstitution experiments indicated that T cell development could be rescued with as little as 9 kb of sequence upstream of *RAG2* (23). Thus, the cis requirements for *RAG* reinduction in DP thymocytes and the functional consequences of reinduction remain poorly defined.

In DP thymocytes V(D)J recombination is targeted to the TCRα locus. The TCRα/δ locus is a 1 megabase locus that contains the δ locus nested between the Vα and the Jα segments; there are 61 Jα segments spread over 70 kb of DNA (24–26). TCRα recombination is believed to begin at the 5′ end of the Jα cluster and progress to the 3′ Jαs during thymocyte maturation (27–29). This idea is indirectly supported by the finding of sterile transcripts emanating from the 5′ T early α promoter (TEA) in late DN thymocytes (30, 31). Successful rearrangement and expression of TCRα genes is marked by an increase in cell surface CD3/TCR levels, but expression of a TCRα/β dimer is not sufficient to turn off *RAG* expression and V(D)J recombination. TCRα recombination and *RAG* expression persist until positive selection (32, 33). Continued TCRα recombination in cells that express nonselected α/β TCRs might result in absence of allelic exclusion, and could theoretically interfere with clonal selection (34). Persistent recombination may nonetheless be advantageous if nonselected or self-reactive receptors are replaced by useful receptors thereby salvaging thymocytes that would otherwise be deleted. Indeed, in transgenic and gene targeted mice, secondary TCRα recombination efficiently replaces TCRs that cannot be positively selected (20, 33, 35–37). Despite the potential importance of secondary TCRα recombination for tolerance and repertoire diversification the extent to which secondary recombination contributes to the TCR repertoire in normal mice has not been determined.

Here we report on T cell development and TCRα recombination in mice that are unable to upregulate *RAG* expression in DP thymocytes. The results indicate that secondary V(D)J recombination makes a major contribution to the normal TCR repertoire.

## Materials and Methods

*Mice.* Clone m3e8A is an 80-kb yeast artificial chromosome (YAC) containing the *RAG1* and *RAG2* genes (RYAC), identified by screening the YAC library of St. Mary's Hospital Medical School (38) with primers specific for *RAG1* (Genethon). YAC DNA was purified by pulsed field electrophoresis as described (39), and microinjected into the pronuclei of fertilized ova of *RAG1*$^{-/-}$ mice (129/SvxCD1 F1) (6). Transgenic founders (RYII and RYIII) were bred for seven generations to C57Bl/6 *RAG1*$^{-/-}$ mice (The Jackson Laboratory). The single-copy line RYII was also bred to *RAG2*$^{-/-}$ mice (Taconic Farms) (7). The *RAG2*$^{-/-}$ TCRβ mice were from Taconic Farms.

*Flow Cytometry.* Antibodies used were: PE anti-CD25, biotin anti-CD44, fluorescein anti-CD3, PE–anti-HSA, PE anti-CD8, allophycocyanin (APC) anti-CD4, APC anti-CD8, PE anti-TCRβ, and APC anti-B220 (BD PharMingen). Biotinylated antibodies were visualized with streptavidin-RED613 (GIBCO BRL). Acquisition and analysis were performed with a FACSCalibur™ and CELLQuest™ (Becton Dickinson). Subpopulations of thymocytes and spleen cells were sorted using a FACS Vantage™ (Becton Dickinson) and final purity was >98%.

*PCR.* For reverse transcription (RT)-PCR total RNA from 50,000 cells from thymus or spleen/lymph nodes was prepared with TRIzol, primed with oligo-dT, and reverse transcribed with Superscript II (GIBCO/BRL). *RAG1* primers were 5′-CAACCAAGCTGCAGACATTCTAGCACTC-3′ and 5′-CACGTCGATCCGGAAAATCCTGGCAATG-3′. β-actin primers were 5′-TACCACTGGCATCGTGATGGACT-3′ and 5′-TTTCTGCATCCTGTCGGCAAT-3′. PCR was performed at 94°C 30 s, 62°C 60 s, 72°C 45 s, for 32 cycles. VJα genes were amplified from cDNA primed with the α chain constant region–specific primer 5′-ATCCATAGCCTTCATGTCCA-3′. PCR was nested using a Cα primer 5′-TCAAAGTCGGTGAACAGGCA-3′ and a degenerate Vα primer AGAAGGTGAAGCAGAGNNM as described (40) for two cycles at 94°C 30 s, 52°C 60 s, 72°C 60 s, followed by 40 cycles at 94°C 30 s, 55°C 60 s, 72°C 60 s. PCR products were cloned before sequencing. V-Jβ sequences were cloned and sequenced using the degenerate Vβ primers 5′-GGMCAYAVTGCTVTKTWCTGGTA-3′, 5′-AAYCATGAYAMMATGTACTGGTA-3′, 5′-CARGCHCCTTCGVTGDNYTGGTA-3′, and Cβ nested primers, 5′-TCAGGCAGTAGCTATAATTGCTCTC-3′, 5′-TTGCCATTCACCCACCAGCTC-3′, and 5′-GCTCAGCTCCACGTGGTCAG-3′. (M = A/C, R = A/G, W = A/T, Y = C/T, K = G/T, V = A/C/G, H = A/C/T, D = A/G/T, N = A/C/G/T). Jα and Vβ-Jβ segments were identified by comparison to the GenBank/EMBL/DDBJ database sequences with accession nos. M64239 and AE000663–5, respectively.

TCRα gene recombination was measured by PCR reactions using the degenerate Vα primer described above in conjunction with either a proximal Jα primer 5′-ACATGAGCTCACTGTCAGCT-3′ (3′ to Jα24.2) or a distal Jα primer 5′-TTACTTGGCTTCACTGTGAG-3′ (3′ to Jα57.9; see Fig. 4). PCR was at: 94°C 30 s, 55°C 60 s, 72°C 3 min for 25 cycles. PCR products were analyzed by electrophoresis in agarose and visualized by blotting and hybridizing with radiolabeled Jα probes 5′-AGGAGGGTCTGCGAAGCTCATCTTT-3′ (proximal) and 5′-ACCAATACAGGCAAATTAACCTTTG-3′ (distal). For single cell PCR, the stained cells and the cytometer sheath fluid were treated with 0.25 pg/ml RNase A to avoid contamination with RNA from lysed cells. Single CD25$^{+}$CD44$^{-}$ DN T cells from RYII*RAG1*$^{-/-}$ and wild-type mice were sorted into 96-well plates containing 4 μl catch buffer (75 mM NaCl, 1 mM DTT, 4 units Promega RNAsin, 7 units Eppendorf Prime RNase inhibitor) per well and placed on dry ice. RT reactions were performed by addition of 7 μl of random hexamer solution (300 ng random hexamers, 2 pmole *RAG1*-specific primer: 5′-CTTGAGTCCCCGATGGGCAGTAAA-3′, 1.4% NP-40, 10 units Eppendorph Prime RNase inhibitor, water) followed by a 1-min incubation at 37°C followed by addition of 14 μl of RT reaction solution (5 μl of 5× first strand Superscript buffer, 1 mM dNTPs, 8 mM DTT, 14 units Promega RNAsin, 7 units Prime RNAsin, 0.5 μl Superscript II [GIBCO BRL]). RT was for 10 min at 25°C, followed by 30 min at 37°C, and the enzyme was destroyed by incubation at 90°C for 6 min. 2.5 μl (10%) of the cDNA for

each cell was used for nested PCR reactions to amplify *RAG1* cDNA or glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a positive control. The primers for both *RAG1* and *GAPDH* were designed to span introns to distinguish cDNA from genomic DNA. PCR primers: *RAG1* external sense: 5′-GCTATCTCT-GTGGCATCGAGTGTT-3′; *RAG1* external antisense: 5′-AAA-GACTTTGGGTTTTAGC-3′; *RAG1*-nested-sense: 5′-GCCG-GGAGGCCTGTGGAG-3′; *RAG1* nested antisense: 5′-CCG-TCGGGTGGATGGAGTCAA-3′; *GAPDH* external sense: 5′-GGTCATCATCTCCGCCCCTTCTG-3′; *GAPDH* external antisense: 5′-CACCCTGTTGCTGTAGCCGTATTC-3′; *GAPDH* nested sense: 5′-TTTGGCATTGTGGAAGGGCT-CAT-3′; *GAPDH* nested antisense: 5′-TCGAAGGTGGAAG-AGTGGGAGTTG-3′. PCR cycle conditions were: 94°C 15 min, 40 cycles of 94°C 30 s, 55°C 30 s, 72°C 30 s, and finally a 7-min extension at 72°C. A total of 138 cells were assayed for *RAG1* expression from the RYII*RAG1*$^{-/-}$ mice and 64 cells were assayed from wild-type mice. To ensure that difference between RYII*RAG1*$^{-/-}$ and control samples was not due to the fourfold decrease in RAG1 mRNA in RYII*RAG1*$^{-/-}$ cells we performed four separate PCR experiments on each RYII*RAG1*$^{-/-}$ cell and diluted control cell cDNA.

*Quantitative Southern Blotting.* Quantitative Southern blotting was performed exactly as described using the Jα19330.11, Jα42417.4, and Jα4.1 probes (28).

*Immunofluorescence.* In situ staining for Nijmegen breakage syndrome (NBS)1 was as described previously (41).

*Online Supplemental Data Section.* The online supplemental data contains two tables, online supplemental Tables S1 and S2. Online supplemental material is available at http://www.jem/org/cgi/content/full/194/4/471/DC1.

## Results

To clarify cis regulation of *RAG* expression in vivo we analyzed transgenic mice carrying an 80-kb YAC containing 33 kb of genomic sequence 5′ of *RAG1* and 12 kb of sequence 5′ of *RAG2* (RYAC; Fig. 1). The two lines reported here (RYII single-copy and RYIII three-copy) were maintained by breeding to *RAG1*$^{-/-}$ or *RAG2*$^{-/-}$ mice and are referred to as RYII*RAG1*$^{-/-}$, RYII*RAG2*$^{-/-}$, and RYIII*RAG1*$^{-/-}$.

*RAG Expression in DN Thymocytes.* To determine whether the RYAC directs expression of *RAG1* and *RAG2* in vivo we measured *RAG1* mRNA levels in CD25$^{+}$ DN thymocytes isolated from wild-type, RYII*RAG1*$^{-/-}$, RYIII*RAG1*$^{-/-}$, and RYII*RAG2*$^{-/-}$ mice by flow cytometry. Steady-state levels of *RAG1* and *RAG2* mRNA were estimated by semiquantitative RT-PCR (Fig. 1). CD25$^{+}$ DN cells isolated from RYII*RAG1*$^{-/-}$, RYIII*RAG1*$^{-/-}$, and RYII*RAG2*$^{-/-}$ mice expressed *RAG* mRNA at three- to fourfold lower levels than wild-type CD25$^{+}$ DN cells (Fig. 1 B). *RAG* indicator expression is variegated in mice that carry BAC transgenes similar to RYAC with a variable fraction of developing lymphocytes expressing the indicator transgene (22). To determine whether only a fraction of the DN cells in RYII*RAG1*$^{-/-}$ and RYIII*RAG1*$^{-/-}$ mice express *RAG1* we conducted single-cell PCR experiments on purified CD44$^{-}$CD25$^{+}$ DN thymocytes. We found that the percentage of CD44$^{-}$CD25$^{+}$ thymocytes expressing

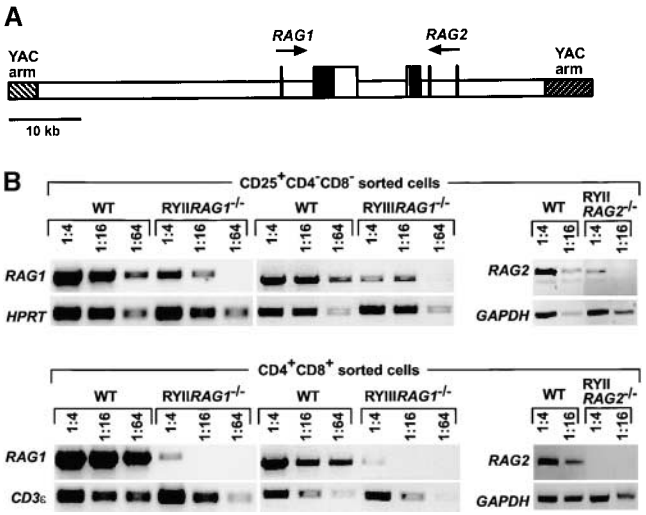



**Figure 1.** *RAG1* is expressed in DN T cells in RYAC transgenic mice. (A) Diagrammatic representation of RYAC an 80-kb YAC containing the *RAG* genes, 12 kb of genomic DNA 5′ of *RAG2*, and 33 kb of genomic DNA 5′ of *RAG1*. (B) *RAG1* expression in FACS® purified thymocyte subpopulations as measured by RT-PCR. *HPRT*, *CD3ε*, or *GAPDH* were used as loading controls for normalizing mRNA. The data shown are representative of five experiments. cDNA dilutions are indicated. WT, wild-type.

*RAG1* in RYII*RAG1*$^{-/-}$ was threefold lower than in wild-type mice (online supplemental Table S1). This heterogeneity in gene expression is consistent with variegation seen in BAC reporter mice that carry similar transgenes (22).

To determine whether the difference in *RAG* expression between RYAC and wild-type mice affects TCRβ recombination and expression we cloned and characterized TCRβ mRNAs from thymus. We find no significant differences in TCRβ V, D, or J usage and no significant differences in the nature of the joints between RYAC and wild-type mice (online supplemental Table S2). We conclude that the pattern of *RAG1* and *RAG2* expression in DN thymocytes in RYAC mice resembles that found in BAC transgenic mice and that this level of expression is sufficient for TCRβ V(D)J recombination.

*RAG Expression in DP Thymocytes.* To determine whether RYAC directs regulated *RAG* expression in DP thymocytes we purified these cells from RYII*RAG1*$^{-/-}$, RYIII*RAG1*$^{-/-}$, RYII*RAG2*$^{-/-}$, and wild-type control mice and measured steady-state levels of *RAG1* and *RAG2* mRNA by semiquantitative RT-PCR (Fig. 1 B). We found that there was little *RAG1* or *RAG2* expression in DP thymocytes in RYII*RAG1*$^{-/-}$, RYIII*RAG1*$^{-/-}$, and RYII*RAG2*$^{-/-}$ mice (60–120-fold less than in wild-type mice in five experiments). Thus, RYII and RYIII transgenic mice resemble previously characterized BAC transgenic mice in that a cis element that is not contained in RYAC is required for regulated *RAG1* and *RAG2* expression in DP thymocytes.

*T Cell Development.* To determine the physiologic consequences of loss of *RAG1* and *RAG2* expression in DP cells we analyzed T cell development in RYII*RAG1*$^{-/-}$,

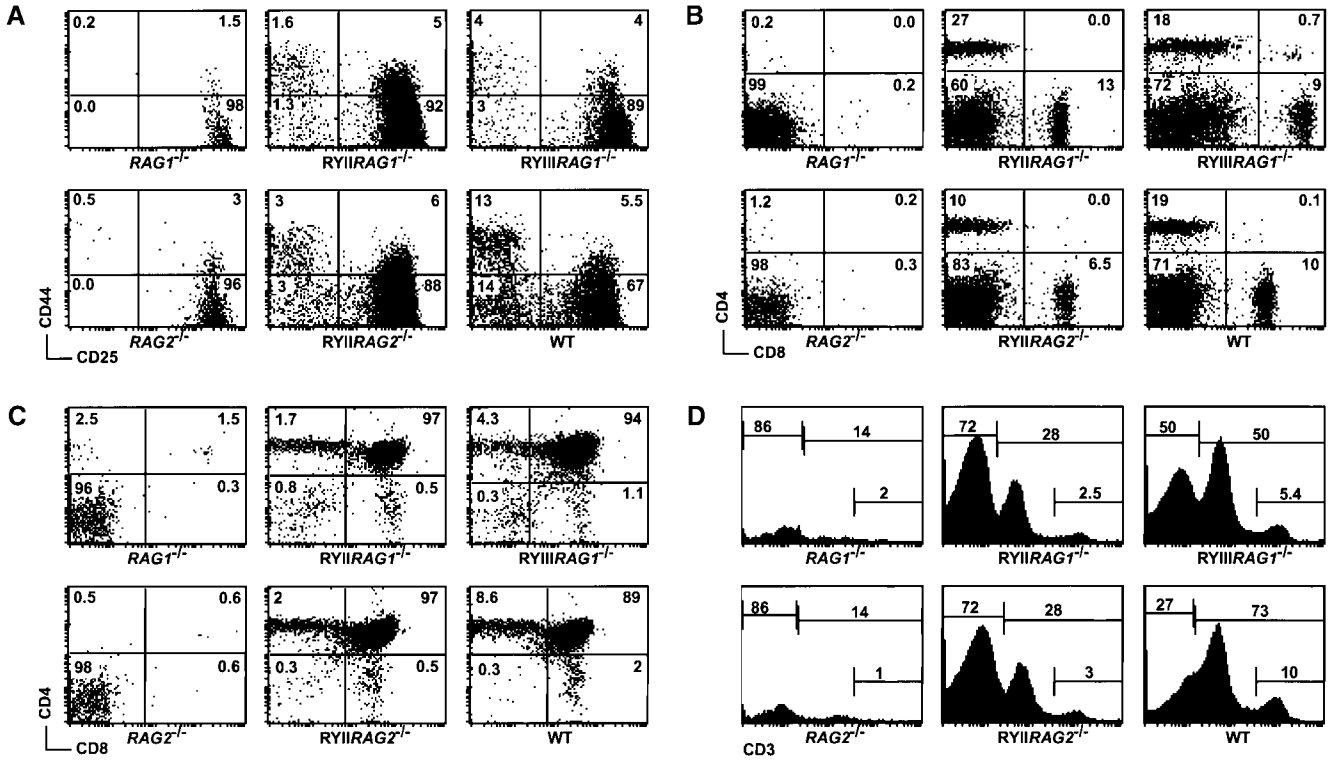



**Figure 2.** T cell development in *RAG1*$^{-/-}$, *RAG2*$^{-/-}$, wild-type (WT), RYII*RAG1*$^{-/-}$, RYII*RAG2*$^{-/-}$, and RYIII*RAG1*$^{-/-}$ mice. (A) CD44 and CD25 staining profiles for DN thymocytes gated on CD4$^{-}$CD8$^{-}$ HSA$^{high}$ cells. (B) CD4 and CD8 staining of splenocytes. (C) CD4 and CD8 staining of thymocytes. (D) CD3 staining of thymocytes. All plots are representative of 2–4 experiments. Numbers show the percentage of cells in each quadrant. The number of SP thymocytes and CD3$^{high}$ cells was 0.5–0.25 of wild-type controls in four separate experiments.

RYII*RAG2*$^{-/-}$, and RYIII*RAG1*$^{-/-}$ mice. We found that the developmental profile of DN cells in RYII*RAG1*$^{-/-}$, RYII*RAG2*$^{-/-}$, and RYIII*RAG1*$^{-/-}$ mice was similar to that of wild-type thymocytes but that the number of thymocytes in the most mature DN subset (CD25$^{-}$CD44$^{-}$) was decreased (Fig. 2 A). There was also a corresponding, small but consistent, increase in the percentage of CD25$^{+}$ CD44$^{-}$ T cells (the immediate precursors of CD25$^{-}$ CD44$^{-}$T cells; Fig. 2 A) and a fourfold decrease in the percentage of these cells in the S or G2/M phase of the cell cycle in RYII*RAG1*$^{-/-}$ and RYIII*RAG1*$^{-/-}$ mice (as measured by analysis of DNA content after DAPI staining; data not shown). The accumulation of nonproliferating CD25$^{+}$ CD44$^{-}$ thymocytes in the transgenic mice resembles the accumulation of these cells in *RAG1*$^{-/-}$ mice and is consistent with variegated *RAG* expression in this stage of T cell development (see above).

Despite the 98–99% reduction in *RAG* expression in DP thymocytes, T cell development in *RAG1*$^{-/-}$ and *RAG2*$^{-/-}$ mice appeared to be reconstituted by the RYAC transgene as measured by the number of cells in the thymus and spleen. In addition, CD4 and CD8 staining profiles in the thymus and the periphery were similar for RYII*RAG1*$^{-/-}$, RYII*RAG2*$^{-/-}$, or RYIII*RAG1*$^{-/-}$ transgenic mice and wild-type controls (Fig. 2, B and C). However, the number of CD4$^{+}$CD8$^{-}$ and CD4$^{-}$CD8$^{+}$ single positive (SP) cells was decreased two- to fourfold in the thymus of RYII*RAG1*$^{-/-}$, RYII*RAG2*$^{-/-}$, and RYIII*RAG1*$^{-/-}$ mice (Fig. 2 C). We conclude that T cell development can proceed to the SP stage with only minimal *RAG* mRNA expression in the DP compartment but fewer SP cells are produced. To examine TCR expression in developing T cells in RYII*RAG1*$^{-/-}$, RYII*RAG2*$^{-/-}$, and RYIII*RAG1*$^{-/-}$ mice we stained thymocytes for ex-

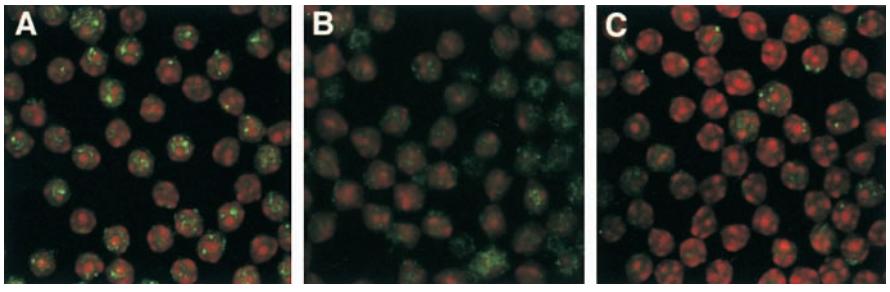



**Figure 3.** NBS1 foci on DP T cells from RYII*RAG1*$^{-/-}$, *RAG2*$^{-/-}$TCRβ transgenic, and wild-type mice. DP thymocytes were stained with anti-NBS1 (green) and counterstained with Topro-3 (red). (A) WT, (B) *RAG2*$^{-/-}$TCRβ, and (C) RYII*RAG1*$^{-/-}$. 460–480 DP cells were examined for each mouse strain for the presence of foci.

pression of the TCR–CD3 complex. Thymocytes can be divided into three groups based on low, medium, and high levels of CD3 expression. $CD3^{low}$ cells are the earliest DP cells and express TCRβ but not TCRα, $CD3^{med}$ cells express TCRα but have not yet completed positive selection, and $CD3^{high}$ cells have completed selection and as a result upregulate surface TCR expression. RYII*RAG1*$^{-/-}$, RYII*RAG2*$^{-/-}$, and RYIII*RAG1*$^{-/-}$ mice showed an altered distribution of CD3 expression: the majority of thymocytes in these mice were $CD3^{low}$ whereas this is only a minor population in the wild-type thymus (Fig. 2 D). In addition, we found that the transgenic mice displayed a two- to fourfold decrease in the number of $CD3^{high}$ cells, which is also consistent with the decrease in the number of SP thymocytes (Fig. 2 C, five experiments). Similar results were obtained by anti-TCRβ staining (not shown). We conclude that decreased *RAG* expression in DP thymocytes in RYII*RAG1*$^{-/-}$, RYII*RAG2*$^{-/-}$, and RYIII*RAG1*$^{-/-}$ mice leads to a decrease in the number of thymocytes expressing medium and high levels of TCR.

*TCRα Recombination.* The relative decrease in *RAG* expression in DP thymocytes and the increase in the percentage of $CD3^{low}$ cells in RYAC mice suggest that there might be lower levels of TCRα recombination. Double stranded DNA break intermediates created during V(D)J recombination at the TCRα locus can be visualized in developing thymocytes by staining nuclei with antibodies to the NBS1 protein (41). To examine the extent of V(D)J recombination in RYII*RAG1*$^{-/-}$ thymocytes directly, we stained purified DP cells from these mice with antibodies to NBS1 and compared them to *RAG2*$^{-/-}$ TCRβ transgenic and wild-type mice (Fig. 3). In agreement with previous results, 25% of wild-type DP thymocytes had NBS1 foci. These foci were not detectable in *RAG2*$^{-/-}$ TCRβ mice, which have normal number of DP cells but do not undergo V(D)J recombination (21). In contrast, 5% of the DP thymocytes from RYII*RAG1*$^{-/-}$ mice showed NBS1 foci. Thus, the percentage of DP thymocytes with double stranded breaks in RYII*RAG1*$^{-/-}$ mice is decreased in a manner consistent with impaired TCRα recombination.

To determine whether the TCRα genes expressed in RYAC transgenic mice differ from the wild-type controls we amplified and sequenced TCRα mRNAs from thymus and spleen (Fig. 4 A). Although the Vα genes expressed in RYII*RAG1*$^{-/-}$ mice and the VJα junctions were indistinguishable from controls, the Jαs used in these mice were highly biased to the proximal end of the Jα cluster (Fig. 4 A, and not shown). Whereas only 8% of the wild-type TCRα genes in the thymus used a Jα from the proximal 10 kb of this cluster, 41% of the TCRα expressed in the RYII*RAG1*$^{-/-}$ thymus used these 5′ most proximal Jαs (42; Fig. 4 A). Further, in wild-type mice 42% of the Jαs were from the distal half of the locus whereas only 7% of the TCRα genes expressed in RYII*RAG1*$^{-/-}$ mice carry Jαs from the distal part of the locus (42; Fig. 4 A). Proximal skewing of the Jαs was even more evident in T cells that had undergone selection and been exported to the spleen. In the wild-type, 75% of spleen T cells expressed TCRαs using distal Jαs whereas only 3% of the TCRαs cloned from RYII spleen T cells used distal Jαs (Fig. 4 A). We conclude that the TCRα genes expressed in RYII*RAG1*$^{-/-}$ mice are highly biased toward proximal Jα usage.

To determine whether skewed Jα usage is due to biased TCRα recombination we measured recombination by PCR using primers specific for the 5′ and 3′ ends of the Jα locus. Both Vα to 5′ Jα and Vα to 3′ Jα rearrangements were readily detected in wild-type mice but only Vα to 5′ Jα rearrangements were found in RYII*RAG1*$^{-/-}$ and RYII*RAG2*$^{-/-}$ mice (Fig. 4 B). Thus, there appears to be a relative absence of recombination to the 3′ Jα locus

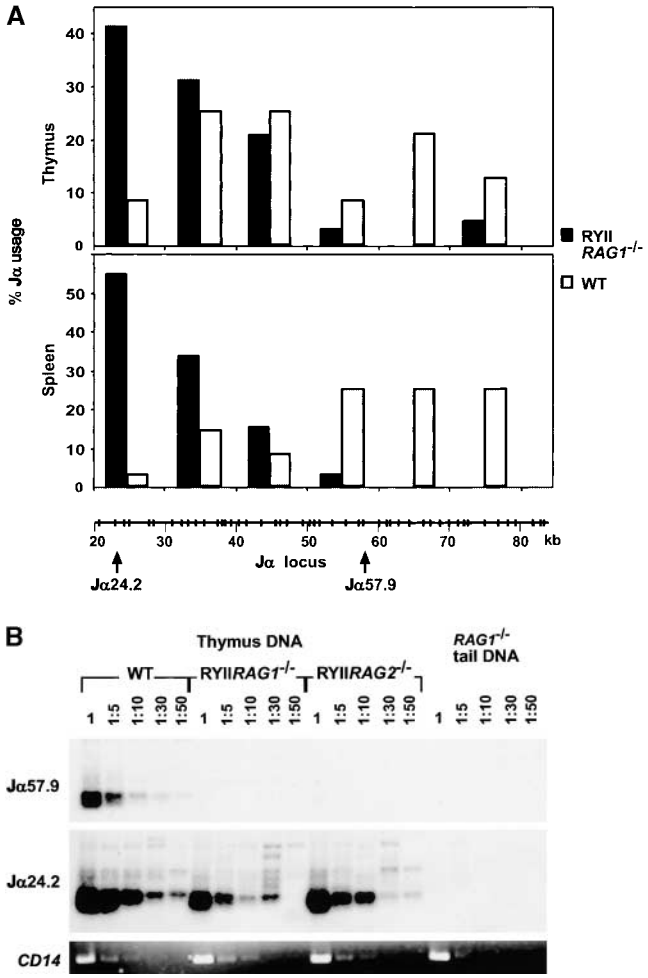



**Figure 4.** 5′ skewed Jα usage in RYII*RAG1*$^{-/-}$ and RYII*RAG2*$^{-/-}$ mice. (A) Percentage of TCRα mRNAs using Jαs found within the indicated 10-kb intervals in the Jα locus. The results represent 68 RYII*RAG1*$^{-/-}$ and 24 wild-type (WT) clones from the thymus, and 33 RYII*RAG1*$^{-/-}$ and 36 WT clones from the spleen. Schematic representation of the Jα locus is shown and position of Jαs from GenBank/EMBL/DDBJ accession no. M64239 with distances in kbs, the position of the PCR primers and probes used in B is shown. (B) V-Jα rearrangement measured by PCR on genomic DNA from total thymus. *RAG1*$^{-/-}$ mouse tail DNA was used as a negative control. The first lane in each case represents ~60 ng of template DNA diluted as shown. PCR on *CD14* was used as loading control. Results are representative of two separate experiments with individual mouse samples.

in RYII*RAG1*$^{-/-}$ and RYII*RAG2*$^{-/-}$ mice. We used Southern blotting on DNA purified from CD3$^{low, med, high}$ thymocytes with probes that hybridize to the 5′, middle, and 3′ ends of the Jα cluster to measure TCRα recombination directly (28). The amount of recombination was standardized with a Cα probe and quantified by phosphorimager analysis (Fig. 5). Overall, RYII*RAG1*$^{-/-}$ and RYIII*RAG1*$^{-/-}$ mice showed less Jα recombination than wild-type controls and almost all of the recombination was restricted to the 5′ portion of the Jα cluster (Fig. 5).

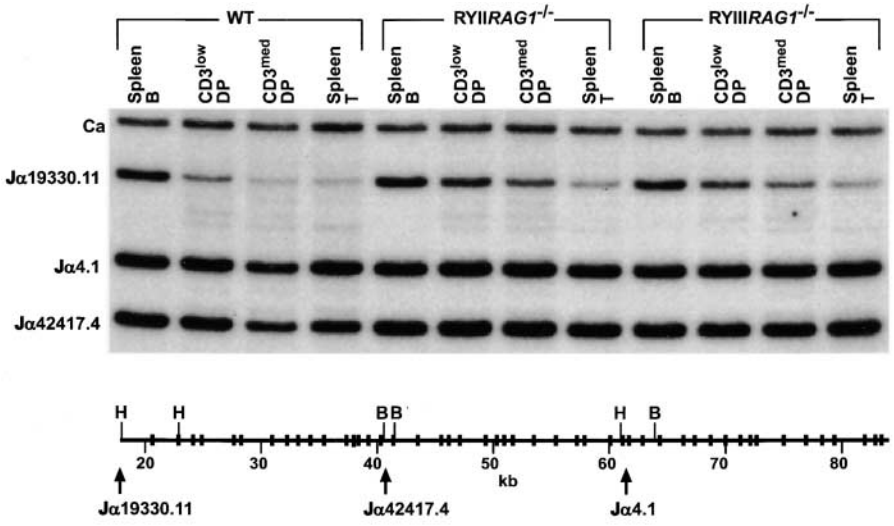
Thymocytes must rearrange and express at least one TCRα gene to become CD3$^{med}$; therefore the theoretical minimum V(D)J recombination that would allow a T cell to become CD3$^{med}$ is 50%. In wild-type mice Jα recombination was detected on 78–80% of the chromosomes in T cells reaching this stage in development, indicating that most CD3$^{med}$ cells have undergone more than a single V(D)J rearrangement (34, 43–45; Fig. 5). In contrast, CD3$^{med}$ thymocytes in RYII*RAG1*$^{-/-}$ and RYIII*RAG1*$^{-/-}$ mice showed only 58 and 57% 5′ Jα recombination. Thus, most CD3$^{med}$ cells in RYII*RAG1*$^{-/-}$ and RYIII*RAG1*$^{-/-}$ mice have only attempted V(D)J recombination on one chromosome.

In wild-type mice TCRα recombination may begin with recombination to 5′ Jαs, and 3′ Jα recombination seems to increase as thymocytes progress to more mature stages in development (28). For example, there was 70% 5′, 29% middle, and 14% 3′ Jα recombination in CD3$^{low}$ DP cells and this increased to 83, 51, and 19%, respectively, in SP cells in wild-type mice (28; Fig. 5, bottom). RYII*RAG1*$^{-/-}$ and RYIII*RAG1*$^{-/-}$ mice showed a more drastic bias to 5′ Jα recombination in CD3$^{low}$ DP thymocytes and there was no significant additional recombination to the middle and 3′ part of the locus as thymocytes progressed in development (Fig. 5). We conclude that RYII*RAG1*$^{-/-}$ and RYIII*RAG1*$^{-/-}$ thymocytes differ from wild-type in that they do not recombine 3′ Jαs in the DP compartment.

## Discussion

*RAG Regulation.* *RAG1* and *RAG2* are closely linked genes that are believed to originate from a transposon which entered the vertebrate lineage at the time of the evolution of jawed fish (2, 4, 5, 46–53). Expression of the RAG nuclease is highly restricted, but the regulation of *RAG* expression remains poorly defined.

In vitro analysis of cis regulation of the *RAG1* promoter revealed only nonspecific basal promoter activity (54–58). In contrast, the *RAG2* promoter displayed preferential activity in lymphoid cell lines that was PAX5 and GATA3 dependent but the activity was not developmentally restricted (59, 60). Two systems have been used to study *RAG* transcription in vivo: *RAG2*$^{-/-}$ blastocyst reconstitution and transgenic reporters (22, 23). In the *RAG2*$^{-/-}$ blastocyst reconstitution experiments an 18-kb genomic fragment extending from 9 kb upstream of the *RAG2* promoter to 2.4 kb downstream of the 3′ UTR was able to re-




| % chromosomal loss | WT | | | RYII*RAG1*$^{-/-}$ | | | RYIII*RAG1*$^{-/-}$ | | |
|---|---|---|---|---|---|---|---|---|---|
| | DP Thymic CD3$^{low}$ | DP Thymic CD3$^{med}$ | Splenic T cells | DP Thymic CD3$^{low}$ | DP Thymic CD3$^{med}$ | Splenic T cells | DP Thymic CD3$^{low}$ | DP Thymic CD3$^{med}$ | Splenic T cells |
| Jα19330.11 | 70 | 78 | 83 | 40 | 58 | 72 | 35 | 57 | 66 |
| Jα42417.4 | 29 | 51 | 51 | 6 | 10 | 13 | 0 | 6 | 0 |
| Jα4.1 | 14 | 32 | 19 | 2 | 13 | 9 | 0 | 0 | 0 |

**Figure 5.** Quantitation of V-Jα rearrangement by Southern blotting. CD4$^+$ CD8$^+$CD3$^{low}$ and CD3$^{med}$ populations were purified from thymus by flow cytometry; splenic T and B cells were purified from spleen. DNA was digested with BamHI (B) and HindIII (H). The probes are 5′ (Jα19330.11), middle (Jα42417.4), 3′ (Jα4.1), and Cα (reference 28). Schematic representation of the Jα locus showing only the relevant restriction sites; the position of the Jα probes is indicated with arrows. Jα hybridization to spleen B cell DNA was used to calculate the relative loss of Jα DNA in purified T cells. The results in the table are from one of two experiments, the variation between experiments was <5%.

constitute T cell development (23). These experiments suggested that all of the information required for *RAG* regulation might be found proximal to *RAG2* (23). In contrast, the transgenic reporter experiments showed that an element in the genomic region 35–70 kb upstream of the *RAG2* promoter is required for *RAG1* and *RAG2* expression in DP T cells (22). Our results with RYAC confirm the presence of a distal regulatory element that regulates both *RAG1* and *RAG2* reinduction and also reconcile the apparent discrepancies between the two sets of in vivo experiments. The RYAC, which has only 12 kb of genomic sequence 5′ of the *RAG2* promoter, resembles the 18-kb fragment used in the $RAG2^{-/-}$ blastocyst system in that both DNA fragments reconstitute the T cell compartment. However, T cell reconstitution by RYAC occurs in absence of the second wave of *RAG* expression in DP T cells. Thus, the distal element identified in the transgenic reporter system could not have been detected by the T cell rescue experiments in the $RAG2^{-/-}$ blastocyst system.

*TCRα Recombination.* In wild-type mice, TCRα recombination is thought to proceed coordinately on both chromosomes without allelic exclusion (34, 43–45, 61). Only a small fraction of the TCRα genes in $CD3^{med}$ DP T cells are in the germline configuration, and there is an increase in the amount of 3′ Jα recombination as thymocytes mature, which is consistent with continuing chromosome loss due to continued TCRα recombination in the DP stage (27, 29). In contrast, in RYAC mice, almost half of the TCRα genes in $CD3^{med}$ DP T cells are in the germline configuration; only 57% of the TCRα alleles are recombined. All $CD3^{med}$ DP T cells must express a TCRα gene, therefore 57% recombination is just over the theoretical limit of 50% required for TCRα expression in these cells. This indicates that most $CD3^{med}$ transgenic T cells undergo TCRα rearrangement on only one allele and suggests that TCRα recombination begins on one chromosome.

The 5′ portion of the TCRα locus is believed to be the first part of the Jα cluster to become available for recombination (27–29). Sterile transcription from the TEA is associated with accessibility to this part of the locus in the late DN stage of T cell development, and in the absence of the TEA the 5′ most Jαs are not recombined (30, 31). The 3′ portion of the Jα cluster is thought to become accessible for recombination later in T cell development in a TEA-independent but TCRα enhancer–dependent fashion (26, 62–64). Thus, the 5′ Jα recombination we find in RYAC transgenic mice might be accounted for by residual RAG protein in thymocytes transiting from the DN to the DP stage and the absence of 3′ Jα recombination due to the relative lack of *RAG* expression in DP thymocytes. Alternatively, the 1–2% of normal levels of *RAG* expression we find in DP T cells could be enough to recombine only 5′ Jα genes. In either case, the finding that only the 5′ Jα genes are rearranged in the absence of the normal second wave of *RAG* expression clearly demonstrates that TCRα recombination is ordered, and that the 5′ side of the Jα cluster is the first to become accessible to the recombinase (27–30).

The second wave of *RAG* expression in DP thymocytes is normally terminated during positive selection (20, 32, 33, 65). Transgenic and gene targeted mice that carry nonselecting receptors undergo persistent TCRα locus secondary recombination (33, 36, 37). Additional support for the idea that there is continuing recombination in DP thymocytes comes from the finding that 3′ Jα rearrangements accumulate as normal thymocytes progress to the mature SP stage (28, 37). RYAC transgenic thymocytes fail to activate the second wave of *RAG* expression and fail to accumulate 3′ Jα rearrangements that are indicative of secondary recombination. Despite this 5′ bias and apparent absence of continued V(D)J recombination, mature RYAC thymocytes show higher levels of Jα chromosome loss than immature $CD3^{low}$ DP thymocytes. We cannot rule out the possibility that the 1–2% of normal level *RAG* expression in RYAC DP thymocytes targets continuing 5′ Jα but not 3′ Jα recombination, but this seems unlikely. It seems more likely that the increase in chromosome loss in mature T cells in RYAC mice simply reflects selection for the few T cells that have randomly recombined both TCRα alleles because these cells have a higher probability of producing an in frame TCRα gene.

By comparing TCRα recombination in wild-type mice with RYAC transgenic mice that do not reinduce *RAG* expression, we can estimate the contribution of secondary TCRα recombination to the normal α/β T cell repertoire. Less than 10% of all TCRα genes expressed in mature RYAC T cells contain Jαs from the 3′ half of the Jα cluster. In contrast, 75% of all TCRα genes expressed by splenic T cells in wild-type mice carry Jαs from the 3′ half of the Jα cluster. Thus, at least 65% of the TCRα genes in wild-type mice appear to be products of secondary recombination, a much higher level than the 25% estimated for Igκ receptor editing in B cells (66). We conclude that the majority of the TCRα repertoire in the spleen of wild-type mice is the product of secondary V(D)J recombination and that secondary recombination makes a major contribution to the normal α/β TCR repertoire.

It has been proposed that γ/δ T cells are the evolutionary precursors of αβ T cells (67). The V(D)J recombination in RYAC α/β T cells is developmentally similar to that of the γ/δ cells in that it is mostly absent in the DP compartment. Unlike γ/δ T cells, in which the rearrangement is not ordered and shows no allelic exclusion (68), the simple pattern of rearrangement in RYAC α/β T cells results in virtual allelic exclusion of the TCRα locus. It leads, however, to a two- to threefold decrease in the number of SP thymocytes and a substantial decrease in the complexity of the α/β T cell receptor repertoire, as only a fraction of the available Jαs are used. We speculate that the cis regulatory element that induces the second wave of *RAG* expression is a late addition to the *RAG* locus that was selected in evolution at the expense of allelic exclusion because this element increased the diversity of the repertoire.

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