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

Nikos Yannoutsos,1 Patrick Wilson,1 Wong Yu,1 Hua Tang Chen,4 Andre Nussenzweig,4 Howard Petrie,3 and Michel C. Nussenzweig1,2

1Laboratory of Molecular Immunology, and 2Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10021
3Immunology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021
4Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Abstract

Assembly of T cell receptor (TCR)αβ 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-TCR 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
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<sup>−/−</sup> 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/β locus is a 1 megabase locus that contains the β 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 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 nonelected 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 TCR 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.

**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<sup>™</sup> and CELLQuest<sup>™</sup> (Becton Dickinson). Subpopulations of thymocytes and spleen cells were sorted using a FACS Vantage<sup>™</sup> (Becton Dickinson) and final purity was >98%.

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’-CAAA-CACCCGAATCTAGCTACCT-3’ and 5’-CAC-TGCATATCCGGAAATCTCTGGCAATG-3’. β-actin primers were 5’-TACCCATGGCATCTGATGAG-3’ and 5’-TTC-TGACATCTGGCAGAAT-3’. PCR was performed at 94°C 30 s, 62°C 60 s, 72°C 45 s, for 32 cycles. Vβ3 genes were amplified from cDNA primed with the α constant region–specific primer 5’-ATCCATAGCTTATGGAACAGCA-3’ and a degenerate Vα primer AGAAGTTGAACAGGNNM (as described) 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. Vβ-Jβ sequences were cloned and sequenced using the degenerate Vβ primers 5’-GOMCAAYAVGCTCTTATGTGA-3’, 5’-AACATGAYMAMATGTACTGTA-3’, 5’-CARGGCHC-CTTGGTVGDNYYTGTA-3’, and Cβ nested primers, 5’-TCAGGCGAGCTTATATAATTGCTTC-3’ and 5’-TTGCCAT-TCAACCCACCTC-3’. PCR products were cloned into pGEM <sup>3’</sup> and sequenced using the degenerate Vα primer 5’-ACATGAGCTTGTAACAGGCA-3’ and a degenerate Vα primer AGAAGTTGAACAGGNNM as described 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. Vβ-Jβ sequences were cloned and sequenced using the degenerate Vβ primers 5’-GOMCAAYAVGCTCTTATGTGA-3’, 5’-AACATGAYMAMATGTACTGTA-3’, 5’-CARGGCHC-CTTGGTVGDNYYTGTA-3’, and Cβ nested primers, 5’-TCAGGCGAGCTTATATAATTGCTTC-3’ and 5’-TTGCCAT-TCAACCCACCTC-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 = G/A/T, N = A/G/C/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’-ACATGAGCTTGTAACAGGCA-3’ (3’ to Jα24.2) or a distal Jα primer 5’-TTACTTTGCCTACTGTGAG-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’-AGGGTCTGGGAAAGTCTACCTT-3’ (proximal) and 5’-ACCAATACAGGCAAATACAATTTC-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<sup>−</sup>/CD44<sup>−</sup> DN T cells from RYIIRAG1<sup>−/−</sup> 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’-CTTC-GAGTCCCCGATGGGCAGTAAA-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 5X 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

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**Materials and Methods**

**Mice.** Clone m3eA 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<sup>−/−</sup> mice (129/SvxCD1 F1) (6). Transgenic founders (RYII and RYIII) were bred for seven generations to C57Bl/6 RAG1<sup>−/−</sup> mice (The Jackson Laboratory). The single-copy line RYII was also bred to RAG2<sup>−/−</sup> mice (Taconic Farms) (7). The RAG2<sup>−/−</sup>/TCRβ mice were from Taconic Farms.
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'-GCTATCTCTT-GTGGCATCGAGTGT-3'; RAG1 external antisense: 5'-AAAGACTTTGTTTTTACGC-3'; RAG1-nested sense: 5'-GCCGGAGGCTGTGGAG-3'; RAG1 nested antisense: 5'-CCGGTCGGGTGATGGATCGA-3'; GAPDH external sense: 5'-GGTCTACATCTCCGGCCCCCTCTG-3'; GAPDH external antisense: 5'-CACCCGGTGTGCTGACGCTTATC-3'; GAPDH nested sense: 5'-TTTGGCCATTGTGGAGAAGGCTCAT-3'; GAPDH nested antisense: 5'-TGGAGTGGAAGAGTGGGAGTG-3'.

Quantitative Southern Blotting. Quantitative Southern blotting was performed exactly as described using the Jn19330.11, Jn12417.4, and Jn4.1 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 RYIIRAG1-/-, RYIII RAG2-/-, and RYIIIRAG1-/-.

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 RYIIIRAG1-/- 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 RYIIIRAG1-/- mice expressed RAG1 mRNA at three- to fourfold lower levels than wild-type CD25+ DN cells (Fig. 1 B). RAG indicator expression is variated 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 RYIIIRAG1-/- mice express RAG1 we conducted single-cell PCR experiments on purified CD4+CD25+ DN thymocytes. We found that the percentage of CD4+CD25+ thymocytes expressing RAG1 in RYIIIRAG1-/- 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-/-, RYIII 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 RYIII RAG2-/- mice (60–120-fold less than in wild-type mice in five experiments). Thus, RYII and RYIII transgenic mouse resembled 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-/-,
RYII\textsuperscript{RAG1}\textsuperscript{−/−}, RYII\textsuperscript{RAG2}\textsuperscript{−/−}, and RYIII\textsuperscript{RAG1}\textsuperscript{−/−} mice. We found that the developmental profile of DN cells in RYII\textsuperscript{RAG1}\textsuperscript{−/−}, RYII\textsuperscript{RAG2}\textsuperscript{−/−}, and RYIII\textsuperscript{RAG1}\textsuperscript{−/−} 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\textsuperscript{RAG1}\textsuperscript{−/−} and RYIII\textsuperscript{RAG1}\textsuperscript{−/−} 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 RYII\textsuperscript{RAG1}\textsuperscript{−/−} 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\textsuperscript{RAG1}\textsuperscript{−/−}, RYII\textsuperscript{RAG2}\textsuperscript{−/−}, or RYIII\textsuperscript{RAG1}\textsuperscript{−/−} 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\textsuperscript{RAG1}\textsuperscript{−/−}, RYII\textsuperscript{RAG2}\textsuperscript{−/−}, and RYIII\textsuperscript{RAG1}\textsuperscript{−/−} mice (Fig. 2 C). We conclude that T cell development can proceed to the SP stage with minimal RAG mRNA expression in the DP compartment but fewer SP cells are produced. To examine TCR expression in developing T cells in RYII\textsuperscript{RAG1}\textsuperscript{−/−}, RYII\textsuperscript{RAG2}\textsuperscript{−/−}, and RYIII\textsuperscript{RAG1}\textsuperscript{−/−} mice we stained thymocytes for expression of TCRβ.

Figure 2. T cell development in RAG1−/−, RAG2−/−, wild-type (WT), RYII\textsuperscript{RAG1}\textsuperscript{−/−}, RYII\textsuperscript{RAG2}\textsuperscript{−/−}, and RYIII\textsuperscript{RAG1}\textsuperscript{−/−} mice. (A) CD44 and CD25 staining profiles for DN thymocytes gated on CD4+CD8− 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.

Figure 3. NBS1 foci on DP T cells from RYII\textsuperscript{RAG1}\textsuperscript{−/−}, 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\textsuperscript{RAG1}\textsuperscript{−/−}. 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. CD3low cells are the earliest DP cells and express TCRβ but not TCRα. CD3med cells express TCRα but have not yet completed positive selection, and CD3high cells have completed selection and as a result upregulate surface TCR expression. RYIIRAG1+/−, RYIIRAG2+/−, and RYIIRAG1−/− mice showed an altered distribution of CD3 expression: the majority of thymocytes in these mice were CD3low 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 CD3high 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 RYIIRAG1+/−, RYIIRAG2+/−, and RYIIRAG1−/− 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 CD3low 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 RYIIRAG1−/− 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 RYIIRAG1−/− mice showed NBS1 foci. Thus, the percentage of DP thymocytes with double stranded breaks in RYIIRAG1−/− 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 RYIIRAG1+/− 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 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 RYIIRAG1−/− 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 RYIIRAG1−/− 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 RYIIRAG1−/− 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 RYIIRAG1−/− and RYIIRAG2+/− mice (Fig. 4 B). Thus, there appears to be a relative absence of recombination to the 3’ Jα locus.
in RYII\textsuperscript{RAG1\textsuperscript{−/−}} and RYII\textsuperscript{RAG2\textsuperscript{−/−}} mice. We used Southern blotting on DNA purified from CD\textsuperscript{3\textlow}\textsuperscript{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 Ca probe and quantified by phosphorimager analysis (Fig. 5). Overall, RYII\textsuperscript{RAG1\textsuperscript{−/−}} and RYII\textsuperscript{RAG1\textsuperscript{−/−}} 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 CD\textsuperscript{3\textmed}, therefore the theoretical minimum V(D)J recombination that would allow a T cell to become CD\textsuperscript{3\textmed} 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 CD\textsuperscript{3\textmed} cells have undergone more than a single V(D)J rearrangement (34, 43–45; Fig. 5). In contrast, CD\textsuperscript{3\textmed} thymocytes in RYII\textsuperscript{RAG1\textsuperscript{−/−}} and RYII\textsuperscript{RAG1\textsuperscript{−/−}} mice showed only 58 and 57% 5′ Jα recombination. Thus, most CD\textsuperscript{3\textmed} cells in RYII\textsuperscript{RAG1\textsuperscript{−/−}} and RYII\textsuperscript{RAG1\textsuperscript{−/−}} 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 CD\textsuperscript{3\textlow} DP cells and this increased to 83, 51, and 19%, respectively, in SP cells in wild-type mice (28; Fig. 5, bottom). RYII\textsuperscript{RAG1\textsuperscript{−/−}} and RYII\textsuperscript{RAG1\textsuperscript{−/−}} mice showed a more drastic bias to 5′ Jα recombination in CD\textsuperscript{3\textlow} 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\textsuperscript{RAG1\textsuperscript{−/−}} and RYII\textsuperscript{RAG1\textsuperscript{−/−}} 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\textsuperscript{−/−} blastocyst reconstitution and transgenic reporters (22, 23). In the RAG2\textsuperscript{−/−} 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-
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 CD3med 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 CD3med DP T cells are in the germline configuration; only 57% of the TCRα alleles are recombined. All CD3med 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 CD3med 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 continuing V(D)J recombination, mature RYAC thymocytes show higher levels of Jα chromosome loss than immature CD3low 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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