Regulation of the TCRα repertoire by the survival window of CD4+CD8+ thymocytes

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T cell receptor (TCR) α alleles undergo primary and secondary rearrangement in double-positive (DP) thymocytes. By analyzing TCRα rearrangement in orphan nuclear receptor RORγ-deficient mice, in which the DP lifespan is shorter, and in Bcl-x_L–transgenic mice, in which the DP lifespan is extended, we show that the progression of secondary V_α to J_α rearrangements is controlled by DP thymocyte survival. In addition, because Bcl-x_L induces a bias towards 3′ J_α usage in peripheral T cells, we conclude that the programmed cell death of DP thymocytes is not simply a consequence of failed positive selection. Rather, it limits the progression of rearrangement along the J_α locus and the opportunities for positive selection, thereby regulating the TCRα repertoire.

The assembly of T cell receptor (TCR) genes occurs at two distinct stages of thymocyte development[1]. Genes encoding the TCRβ chain undergo variable, diversity and joining, or V(D)J, recombination at the $CD4^-CD8^-$ double negative (DN) stage. Successful TCRβ rearrangement and the pairing of TCRβ with pTα signals to DN thymocytes that they should proliferate and differentiate to the $CD4^+CD8^+$ double positive (DP) stage. Rearrangement of genes encoding the TCRα chain at the DP stage has several distinctive features. First, initial rearrangement of TCRα utilizes J_α segments at the 5′ end of the locus[2–4]. Second, V_α to J_α rearrangement occurs on both alleles due to an apparent lack of allelic exclusion[5]. Third, the use of more 3′ J_α segments occurs through a process of V_α to J_α secondary rearrangement that replaces the primary $V_\alpha J_\alpha$[6,7]. Fourth, rearrangement is coordinated so that nearby J_α segments are used on the two alleles[8–11]. V_α to J_α rearrangement can be terminated by positive selection, which induces the down-regulation of recombination-activating gene 1 (RAG-1) and RAG-2[12–14]. However, if the primary rearrangement fails, secondary rearrangements can test additional V_α segments located 5′ and additional J_α segments located 3′ to the primary $V_\alpha J_\alpha$[7,10]. Secondary rearrangement plays a key role in the formation of a normal TCR repertoire; DP thymocytes with a limited ability to undergo secondary rearrangement, due to defective RAG-1 or RAG-2 expression, have an unusually 5′-biased J_α repertoire[15]. However, the role played by secondary rearrangement in the formation of the TCR repertoire has been challenged[8].

Two *cis*-acting elements, the T early α (TEA) promoter and the TCRα enhancer (Eα), regulate TCRα rearrangement[16,17]. Mice lacking Eα show a profound defect in TCRα recombination and expression that is associated with impaired chromatin structure across the entire J_α locus[17,18]. It has been suggested that the TEA promoter—which is located immediately 5′ to the J_α cluster—is a "rearrangement-focusing" element, whose role is to force the initiation of TCRα rearrangements to the 5′ end of J_α locus[16]. Mice lacking TEA and its promoter show defective recombination that involves the nine most 5′ J_α segments[16].

The orphan nuclear receptor RORγ (retinoic acid receptor–related orphan receptor) and its thymus-specific isoform RORγt are transcription factors that play critical roles in thymocyte development[19,20]. RORγ and RORγt differ from each other only in their 5′ exons, due to alternative promoter usage[19,20]. Both isoforms are expressed at their highest amounts in DP thymocytes. Mice that lack, due to a deletion of the shared DNA-binding domain, both RORγ and RORγt (which are referred to hereafter as $ROR\gamma^{-/-}$ mice) show increased programmed cell death of DP thymocytes[21,22]. The production of mature $CD4^+$ and $CD8^+$ single-positive (SP) thymocytes is reduced by ~90%[21,22]. The massive apoptosis of $ROR\gamma^{-/-}$ DP thymocytes is due to reduced expression of the anti-apoptotic protein Bcl-x_L and can be corrected by expression of Bcl-x_L as a transgene[21]. Normal thymocyte maturation also depends on the down-regulation of RORγt subsequent to the DP stage[23].

It has been suggested that RORγt is a regulator of TCRα gene recombination on the basis of its binding to the TEA promoter *in vitro*[24]. To investigate whether RORγ and/or RORγt regulate TEA promoter activity and TCRα rearrangement *in vivo*, we examined the J_α repertoire in $ROR\gamma^{-/-}$ mice. We show here that in contrast to J_α usage in $TEA^{-/-}$ mice, 5′ J_α segments were over-represented and 3′ segments under-represented in $ROR\gamma^{-/-}$ mice. In addition, the impaired 3′ J_α usage in $ROR\gamma^{-/-}$ mice was corrected by expression of Bcl-x_L as a transgene. Transgenic expression of Bcl-x_L not only corrected defective 3′ J_α usage in the thymocytes of $ROR\gamma^{-/-}$ mice, but also skewed J_α usage to the very

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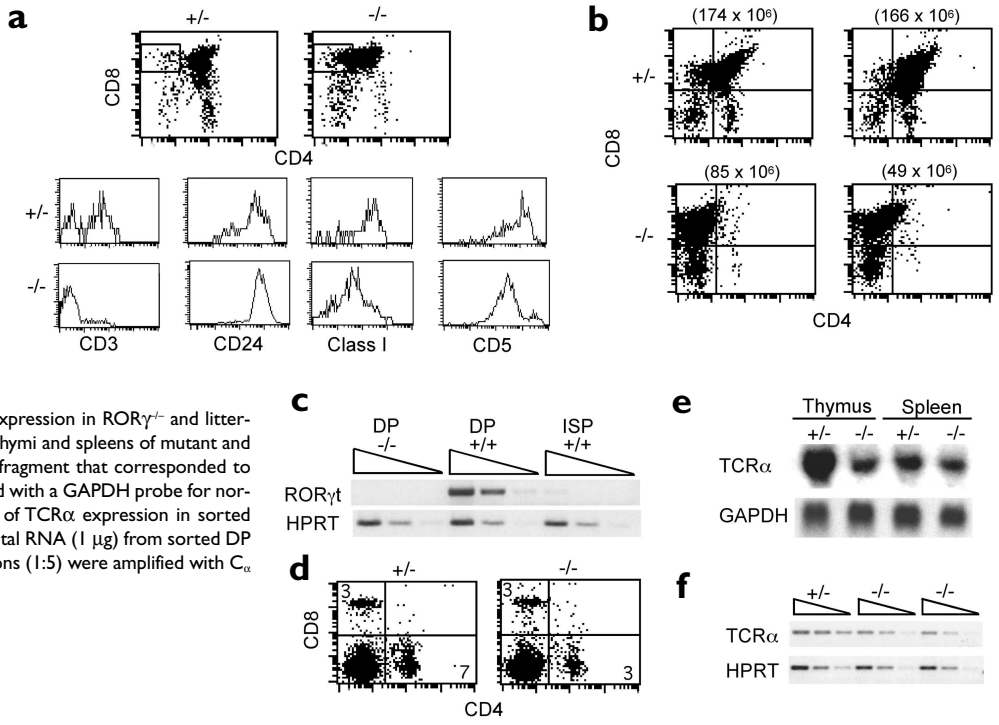


Figure 1. RORγ and/or RORγt promote thymocyte differentiation from the ISP to the DP stage. (**a**) FACS analysis of thymocytes from 3-week-old RORγ$^{-/-}$ (–/–) and littermate control (+/–) mice. CD8$^+$ SP thymocytes were gated and further analyzed for the expression of CD3, CD5, CD24 and MHC class I. (**b**) CD4 and CD8 FACS profiles of thymi from two 9-day-old RORγ$^{-/-}$ and two littermate control mice, with the total number of thymocytes shown in parentheses. (**c**) Semi-quantitative RT-PCR analysis of RORγt expression in ISP thymocytes. Serial dilutions (1:5) were amplified with RORγt and HPRT primers. (**d**) CD4 and CD8 FACS profiles of spleen from the same mice used in **a**. Numbers denote the percentage of cells in each gate. (**e**) Northern blot analysis of TCRα expression in RORγ$^{-/-}$ and littermate control mice. Total RNA (10 μg) from the thymi and spleens of mutant and control mice were probed with a 400-bp DNA fragment that corresponded to exon I of C_α. The blot was stripped and reanalyzed with a GAPDH probe for normalization. (**f**) Semi-quantitative RT-PCR analysis of TCRα expression in sorted DP thymocytes from mutant and control mice. Total RNA (1 μg) from sorted DP thymocytes was reverse-transcribed. Serial dilutions (1:5) were amplified with C_α and HPRT primers.

3′ end of the locus in both RORγ$^{-/-}$ and wild-type mice. Bcl-x_L expression also induced a 3′ J_α bias to the peripheral TCRα repertoire. These results show that the programmed cell death of DP thymocytes is a key parameter that limits the progression of rearrangements along the J_α locus and regulates the repertoire of positively selected T cells.

Results

Impaired ISP to DP transition in RORγ$^{-/-}$ mice

RORγ$^{-/-}$ mice have a 60–80% reduction of DP thymocytes and a >90% reduction of CD4$^+$ SP thymocytes compared to littermate controls[21,22]. The reduction in DP thymocytes may result from increased cell death due to an almost complete absence of Bcl-x_L protein expression[21]. Although CD8$^+$ SP cells represent 3–5% of thymocytes in normal mice, they represented 25–30% of thymocytes in RORγ$^{-/-}$ mice[21] (**Fig. 1a**). This abnormal CD8$^+$ SP population may reflect a skewed production of mature CD8$^+$ SP cells. Alternatively, thymocyte development may be impaired at the CD8$^+$CD4$^-$CD3$^-$ immature single-positive (ISP) stage. To distinguish between these possibilities, we analyzed this population using a three-color fluorescent-activated cell sorter (FACS). Most of the CD8$^+$ SP thymocytes from RORγ$^{-/-}$ mice were CD3$^-$CD24^{hi}MHC class I^{lo}CD5^{lo}, a phenotype that is characteristic of ISP thymocytes (**Fig. 1a**). The number of ISP cells in the thymi of 3–4-week-old RORγ$^{-/-}$ mice was increased fivefold compared to littermate controls ($9.8\pm1.4\times10^6$ (n=3) *versus* $2.0\pm0.8\times10^6$ (n=3)). In addition, the ISP to DP transition was delayed in 9-day-old RORγ$^{-/-}$ mice (**Fig. 1b**). These results show that thymocyte development is inhibited at the ISP stage in RORγ$^{-/-}$ mice and suggest a role for RORγt and/or RORγ in promoting thymocyte maturation from the ISP to the DP stage. We found that RORγt was expressed in low amounts in ISP cells, but was up-regulated ~25-fold in DP thymocytes (**Fig. 1c**). Therefore, up-regulation of RORγt in DP thymocytes is likely to be important for an efficient ISP to DP transition.

We also analyzed mature T cell populations in the spleens of RORγ$^{-/-}$ mice (**Fig. 1d**). RORγ$^{-/-}$ mice (3–6 weeks old) had a slightly higher number of total splenocytes than littermate controls ($207\pm37\times10^6$ (n=5) *versus* $162\pm29\times10^6$ (n=6), respectively). Young RORγ$^{-/-}$ mice (3 weeks old) had a slightly reduced CD4$^+$ T cell population (7×10^6 *versus* 10×10^6 cells) (**Fig. 1d**), which was likely due to diminished production in the thymus. By 4 weeks of age, splenic CD4$^+$ T cell numbers in RORγ$^{-/-}$ mice were comparable to those in littermate controls (data not shown). Thus, perturbations in the mature T cell compartment of RORγ$^{-/-}$ mice appeared minimal with this analysis.

The reduced production of mature SP thymocytes in RORγ$^{-/-}$ mice may reflect a defect in the expression of TCRα genes because RORγt is predominantly expressed at the DP stage[25]. We examined the steady-state level of TCRα transcripts by RNA blot analysis. Total thymocytes of RORγ$^{-/-}$ mice expressed approximately one-third as much TCRα mRNA as thymocytes from littermate controls (**Fig. 1e**). However, splenocytes of RORγ$^{-/-}$ mice expressed similar amounts of TCRα mRNA compared to controls (**Fig. 1e**). Reduced TCRα mRNA expression in unfractionated thymocytes could simply have been due to a reduced DP population. To test this, we sorted DP thymocytes from both RORγ$^{-/-}$ and littermate control mice and performed a semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR) to determine TCRα mRNA expression. The expression of TCRα mRNA in RORγ$^{-/-}$ DP thymocytes was reduced to 20–30% that of control DP thymocytes (**Fig. 1f**). Thus, RORγt and/or RORγ may regulate the expression of TCRα transcripts in DP cells. Taken together, these data show that RORγt and/or RORγ play critical roles at multiple stages of thymocyte development. In addition, they suggest that the diminished DP and SP thymocyte populations in RORγ$^{-/-}$ mice result from both the increased death of DP cells[21,22] and decreased differentiation of ISP thymocytes to the DP stage.

Impaired 3′ TCR J_α usage in RORγ$^{-/-}$ mice

Mice that lack TEA and its promoter show a defective rearrangement of V_α segments to J_α61 through J_α53[16]. To determine whether RORγt and/or RORγ regulate TEA promoter activity *in vivo*, we examined J_α usage in splenocytes from RORγ$^{-/-}$ mice using a method that has been

described[16]. Analysis of total splenocytes was valid because CD4$^+$ and CD8$^+$ SP T cells were comparably represented in the spleens of adult mutant and control mice. TCRα transcripts from splenocytes were reverse-transcribed and PCR-amplified with primers specific for either of the two V_α families (V_α8 or V_α3) along with a C_α primer[16]. PCR products were then analyzed by Southern blotting with a series of J_α-specific oligonucleotide probes to assess J_α usage, and an internal C_α probe was used to assess the quantity of total V_α-C_α PCR products (**Fig. 2a**).

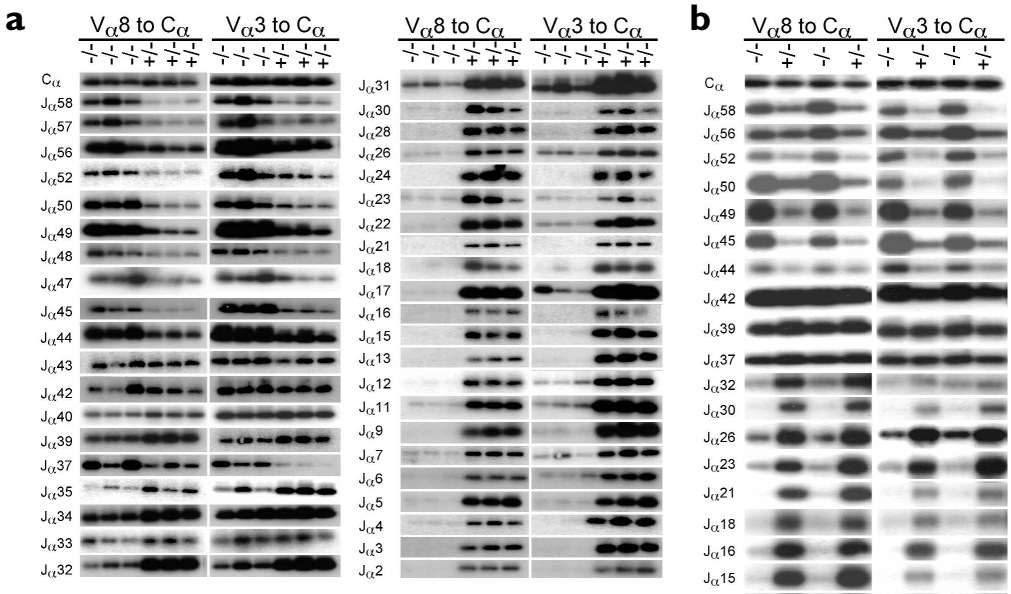


Figure 2. Impaired 3′ J_α usage in ROR$\gamma^{-/-}$ mice. (**a**) Profiles of J_α usage in the spleens of three pairs of ROR$\gamma^{-/-}$ and littermate control mice, aged 3, 6 and 8 weeks old. V_α-C_α RT-PCR was done with primers to V_α 8 and C_α or V_α3 and C_α. PCR products were blotted and hybridized with the indicated J_α-specific oligonucleotide probes. An internal C_α oligonucleotide was used for normalization of the template. (**b**) Profiles of J_α usage in the thymi of two pairs of ROR$\gamma^{-/-}$ and littermate control mice, aged 3 and 6 weeks old.

Comparisons of J_α usage in ROR$\gamma^{-/-}$ and control T cells revealed the following. First, the most 5′ J_α segments, 58 through 44, were over-represented by up to fivefold in ROR$\gamma^{-/-}$ mice compared to controls. This contrasted with the phenotype observed for TEA$^{-/-}$ mice, in which usage of the first nine J_α segments is reduced[16], and argues against an essential role played by RORγt and/or RORγ in regulating TEA promoter activity *in vivo*. In contrast, J_α segments 43 to 34 were used comparably in mutant and control mice, whereas usage of segments 33 through 31 was moderately reduced in ROR$\gamma^{-/-}$ mice (**Fig. 2a**). Usage of J_α30 through J_α2 was much reduced in ROR$\gamma^{-/-}$ mice (**Fig 2a**). Thus, the 3′ J_α segments are underused and 5′ segments are correspondingly overused in ROR$\gamma^{-/-}$ mice. Analysis of J_α usage in ROR$\gamma^{-/-}$ thymocytes gave similar results (**Fig. 2b**). Defective 3′ J_α usage in ROR$\gamma^{-/-}$ mice is not age-related because similar results were obtained from mutant and littermate control mice that ranged in age from 3 to 8 weeks (**Fig. 2a**). These data suggest that RORγt and/or RORγ are essential for expression and/or recombination in the 3′ half of the J_α cluster.

Defective V_α to J_α recombination in ROR$\gamma^{-/-}$ mice

The lack of 3′ J_α usage in ROR$\gamma^{-/-}$ mice could result from a defect in V_α-J_α recombination. Alternatively, DP thymocytes that express TCRs with the use of 5′ J_α segments might preferentially undergo positive selection and maturation in the absence of RORγt and/or RORγ. To distinguish between these possibilities, we directly examined V_α to J_α recombination using genomic DNA from sorted DP thymocytes and long-range PCR[26]. A V_α3 family primer was used in combination with each of eight J_α primers to amplify genomic fragments generated by V_α3 to J_α rearrangement. PCR products were then probed with a radiolabeled oligonucleotide specific for the J_α immediately 5′ to that used in PCR amplification. This method allows direct visualization of recombination events between a V_α and a set of J_αs that extend 5′ from the J_α used as a probe. Recombination between V_α3 and 5′ J_α segments was readily detected in ROR$\gamma^{-/-}$ mice, as assessed with primers for J_α segments 56, 42 and 35 (**Fig. 3a**). In contrast, recombination between V_α3 and 3′ J_α segments was reduced or completely absent, as reflected by the lack of PCR products when primers for J_α segments 27, 17, 12, 7 and 4 were used (**Fig. 3a**). These results show that the lack of 3′ J_α usage in ROR$\gamma^{-/-}$ mice is due to a defect in V_α to J_α recombination.

Mice with defective RAG-1 or RAG-2 re-induction in DP thymocytes show a 50–70% reduction in mature CD4$^+$CD8$^-$ and CD8$^+$CD4$^-$ cells[15]. In addition, similar to our observation in ROR$\gamma^{-/-}$ mice, TCRα rearrangement is restricted to 5′ J_α segments[15]. To determine whether the lack of TCRα rearrangement to 3′ J_α segments in ROR$\gamma^{-/-}$ mice is due to a lack of RAG expression in DP thymocytes, we examined the expression of RAG-1 and RAG-2 mRNA in sorted DP thymocytes using semi-quantitative RT-PCR. ROR$\gamma^{-/-}$ DP thymocytes showed only a modest reduction in RAG-1 and RAG-2 expression (**Fig. 3b**).

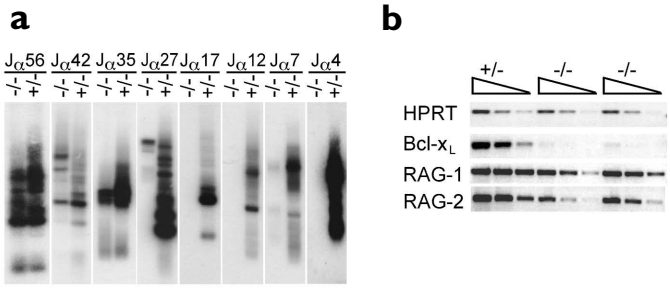


Figure 3. Impaired 3′ J_α usage in ROR$\gamma^{-/-}$ mice is due to defective V_α to J_α recombination. (**a**) V_α-J_α rearrangement was assessed by long-range PCR. Genomic DNA (100 ng) taken from the sorted DP thymocytes of mutant and control mice was subjected to PCR with primers to the V_α3 family and specific J_α segments, as indicated. PCR products were visualized by hybridization with an oligonucleotide probe specific for the J_α immediately 5′ to that used for amplification. No PCR products were amplified from mouse tail DNA, which served as a negative control (data not shown). (**b**) Expression of Bcl-x_L, RAG-1 and RAG-2 mRNA in the DP thymocytes of ROR$\gamma^{-/-}$ and control mice. Total RNA from the sorted DP thymocytes of one control and two different mutant mice were subjected to semi-quantitative analysis for the expression of indicated molecules as in Fig. 1. HPRT served as a cDNA template control.

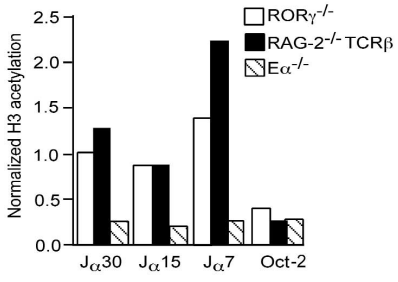


Figure 4. Histone H3 acetylation status of 3′ J_α chromatin. ChIP assays were performed on formaldehyde cross-linked chromatin isolated from DP thymocytes from RORγ$^{-/-}$, RAG-2$^{-/-}$ TCRβ Tg and Eα$^{-/-}$ mice. Following blot hybridization and phosphorimager quantification, normalized H3 acetylation values were determined with the equation: (bound/input for test site)/(bound/input for actin).

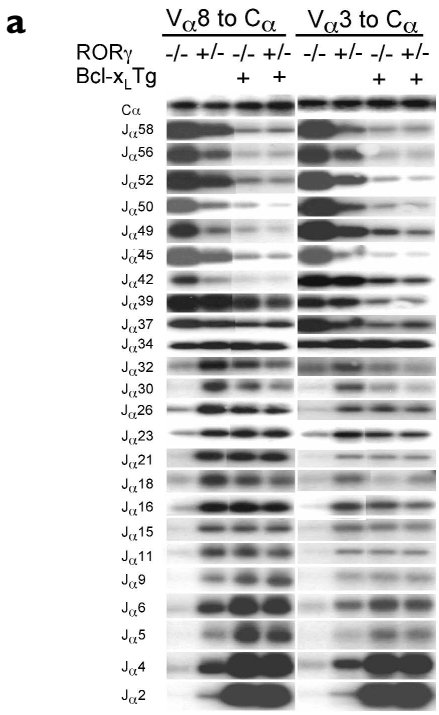
In contrast, Bcl-x_L expression was much reduced, in agreement with published data[21,22]. These results suggest that the lack of TCRα rearrangement involving 3′ J_α segments is unlikely to be caused by defective RAG-1 and RAG-2 re-induction in RORγ$^{-/-}$ mice.

J_α chromatin structure in RORγ$^{-/-}$ mice

V(D)J recombination is developmentally regulated through modulation of chromatin accessibility[17,27]. The histone acetylation status of TCR locus chromatin correlates well with TCR gene accessibility and can influence TCR gene rearrangement[18,28,29]. One possible explanation for the lack of 3′ J_α recombination in RORγ$^{-/-}$ mice is that RORγt and/or RORγ may regulate chromatin accessibility by binding to a promoter in the 3′ portion of the J_α locus. We identified a potential binding site (AGGTCA preceded by an AT-rich region) for RORγt and/or RORγ that was located 197-bp upstream of J_α30 and found that RORγt specifically bound to this element in *in vitro* gel-shift assays (data not shown). This result prompted us to examine whether RORγt and/or RORγ regulates the chromatin structure of 3′ J_α segments. To do so, we tested the acetylation status of histone H3 within the 3′ half of the J_α locus using a chromatin immunoprecipitation (ChIP) assay. Chromatin from the sorted DP thymocytes of RORγ$^{-/-}$ mice was immunoprecipitated with antibody specific for acetylated histone H3 and immunoprecipitated DNA was analyzed by quantitative PCR. Unfractionated thymocytes from Eα$^{-/-}$ mice were used as negative controls and unfractionated thymocytes from RAG-2$^{-/-}$ mice that expressed a rearranged TCRβ transgene were used as positive controls. These comparisons were valid, as >95% of thymocytes in the Eα$^{-/-}$ and RAG-2$^{-/-}$ TCRβ transgenic (Tg) mice are DP[17,30] and because all three thymocyte preparations should be homogeneously unrearranged across the 3′ portion of the J_α locus. Acetylation of 3′ J_α segments of RORγ$^{-/-}$ mice was increased compared that in the Eα$^{-/-}$ mice and was comparable to that in RAG-2$^{-/-}$ TCRβ Tg mice (**Fig. 4**). In comparison, the B lymphocyte–specific gene encoding Oct-2 was hypoacetylated in the same samples (**Fig. 4**). These results argue against a role for RORγt and/or RORγ in regulating the acetylation status of 3′ J_α chromatin *in vivo* and indicate that 3′ J_α acetylation status cannot account for the impaired rearrangement of 3′ J_α segments in RORγ$^{-/-}$ mice.

J_α usage determined by the survival of DP cells

It has been proposed that TCRα recombination will continue until DP thymocytes are positively selected or undergo programmed cell



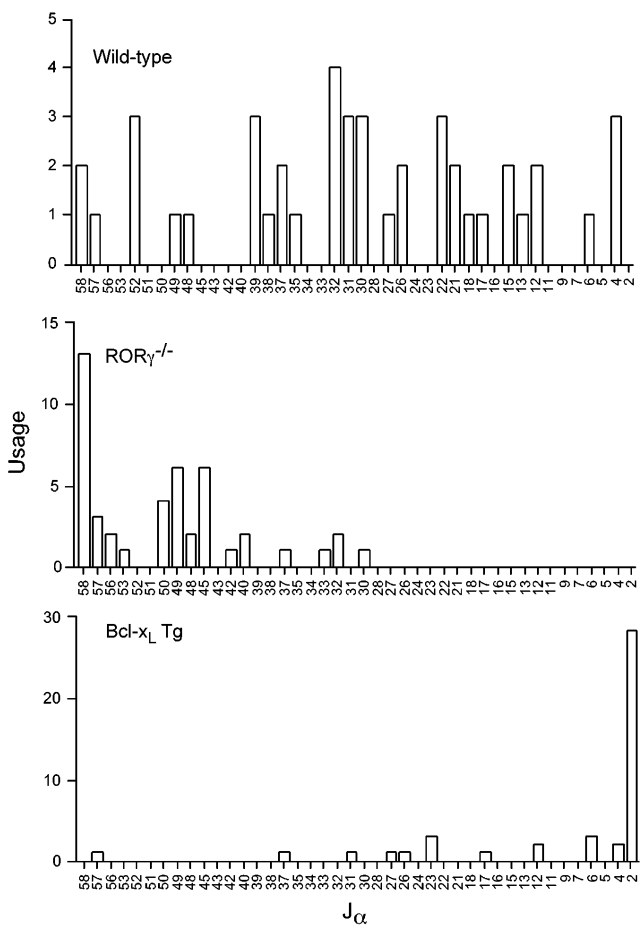


Figure 5. Bcl-x_L expression in DP thymocytes promotes 3′ J_α usage in thymus. (**a**) Profiles of J_α usage in the thymus of RORγ$^{-/-}$, RORγ$^{+/-}$, RORγ$^{-/-}$ Bcl-x_L Tg and Bcl-x_L Tg mice, as determined by hybridization. V_α-C_α RT-PCR was performed with primers to V_α8 and C_α or V_α3 and C_α. PCR products were blotted and hybridized with the indicated J_α-specific oligonucleotide probes. An internal C_α oligonucleotide was used for normalization of the template. (**b**) J_α usage in the thymi of RORγ$^{-/-}$, RORγ$^{+/-}$ and Bcl-x_L Tg mice, as determined by sequence analysis. V_α8 to C_α PCR products were cloned and J_α usage was determined by sequencing. The number of appearances of individual J_α segments is plotted.

death[6]. Given the massive apoptosis of DP thymocytes in RORγ$^{-/-}$ mice, the lack of 3′ J_α usage in these mice may be due to a shortened DP lifespan that prevents recombination events from progressing to the 3′ end of the locus. To test this, we examined J_α usage in the thymi of RORγ$^{-/-}$ mice that were expressing a Bcl-x_L transgene under the control of the *lck* proximal promoter[21]. In these mice, Bcl-x_L expression is increased tenfold compared to wild-type controls and prevents the massive apoptosis of RORγ$^{-/-}$ DP thymocytes[21]. The total number of thymocytes in RORγ$^{-/-}$ Bcl-x_L Tg mice is similar to that of Bcl-x_L Tg mice and is approximately twofold more than in wild-type controls[21]. Bcl-x_L transgene expression in RORγ$^{-/-}$ mice not only corrected the defect in 3′ J_α usage, but also heavily skewed J_α usage to the very 3′ end of the locus (**Fig. 5a**). Hybridization signals for J_α2 in RORγ$^{-/-}$ mice were at least tenfold higher than in wild-type controls, whereas hybridization signals for J_α58 through J_α45 were reduced (**Fig. 5a**). A similar skewing was observed in mice that expressed the Bcl-x_L transgene on a wild-type background (**Fig. 5a**).

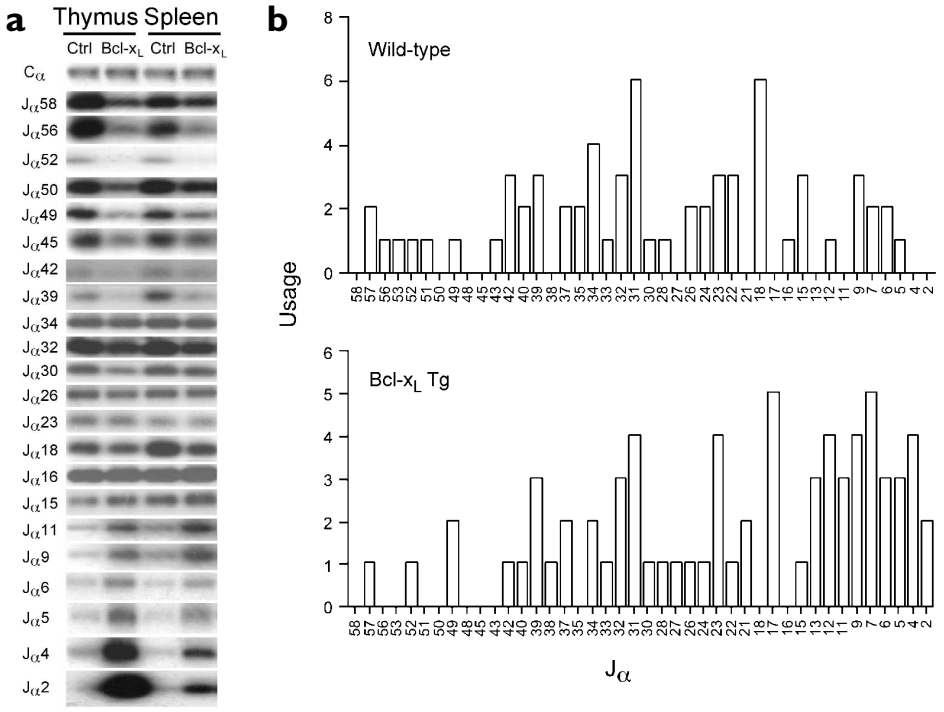


Figure 6. Bcl-x_L expression in DP thymocytes promotes 3′ J_α usage in splenocytes. (**a**) Profiles of J_α usage in splenocytes of Bcl-x_L Tg and normal control mice, determined by hybridization of V_α8-C_α PCR products as in Fig. 5a. Thymocytes from control and Bcl-x_L Tg mice were included for direct comparison. (**b**) J_α usage in splenocytes of Bcl-x_L Tg and normal mice, determined by sequence analysis of V_α8 to C_α PCR products as in Fig. 5b.

The above analysis addressed relative changes in J_α usage between mutant and control mice but did not reveal actual differences in usage among different J_α segments. To evaluate this, we subcloned and sequenced V_α8 to C_α RT-PCR products prepared from thymocyte mRNA samples. Wild-type V_α8-C_α transcripts contained J_α segments distributed evenly across the J_α locus (**Fig. 5b**). In contrast, J_α segments downstream of J_α30 were detected in 0/45 clones that represented V_α8-C_α transcripts from RORγ$^{-/-}$ mice but were detected in 41/44 clones that represented V_α8-C_α transcripts from Bcl-x_L Tg mice (**Fig. 5b**). In addition, most clones from Bcl-x_L Tg mice (64% compared to 0% in wild-type) used J_α2, the last expressed J_α[31]. These results directly show that the survival window of DP thymocytes influences how far V_α-J_α recombination events can proceed along the J_α locus. The preponderance of J_α2 rearrangements in Bcl-x_L Tg thymocytes likely reflects the fact that secondary rearrangements reach the 3′ end of the J_α array in a greater proportion of thymocytes in these mice and that those thymocytes in which rearrangements reach the end of the J_α array are not quickly eliminated by programmed cell death.

Peripheral J_α usage in Bcl-x_L Tg mice

If secondary rearrangements proceed to the 3′ end of the J_α locus with greater frequency in Bcl-x_L Tg mice, there might be a coordinated increase in the frequency with which thymocytes using more 3′ J_α segments are positively selected and exported to the periphery. Indeed, hybridization analysis indicated a 3′ skewing in the spleen that was similar to that in the thymus (**Fig. 6a**). One exception was J_α2: although it increased in Bcl-x_L Tg compared to control spleens, it was not as markedly over-represented as it was in thymi. To address splenic J_α usage in greater detail, we subcloned and sequenced V_α8 to C_α RT-PCR products prepared from splenic mRNA samples. Wild-type V_α8-C_α transcripts contained J_α segments that were distributed evenly across the J_α locus (**Fig. 6b**). Of the 65 clones analyzed, 34 (52%) used J_αs located 5′ of J_α30, whereas 31 (48%) used more 3′ J_α segments. In contrast, J_α usage in splenocytes from Bcl-x_L Tg mice was skewed to the 3′ end. Of the 71 clones analyzed, only 22 (31%) used J_α segments located 5′ of J_α30, and 49 (69%) used more 3′ segments. In addition, more than half the splenic T cells in Bcl-x_L Tg mice used J_αs 17 to 2, whereas only one-fifth of control splenic T cells used the same J_α segments. Taken together, these results demonstrate that the survival window of DP thymocytes influences the peripheral TCRα repertoire and suggest that many thymocytes die before they have a chance to test and positively select TCRs using the most 3′ J_α segments. That J_α2 is so heavily over-represented in the thymus but not the periphery of Bcl-x_L Tg mice is consistent with these thymocytes having failed all attempts at positive selection.

Impaired 5′ V_α usage in RORγ$^{-/-}$ mice

It is believed that secondary TCRα rearrangement is carried out in a bidirectional and coordinated fashion in which rearrangements proceed 5′→3′ through the J_α array and 3′→5′ through the V_α array. RORγ$^{-/-}$ mice provide a good *in vivo* system with which to test this, and we predicted that in RORγ$^{-/-}$ mice V_αs located at the 5′ end of TCRα locus would not be utilized due to a shortened DP lifespan. We therefore examined usage of V_α19 at the very 5′ end of the TCR V_α locus[32] by semi-quantitative RT-PCR. V_α19 usage was not detected in the thymi of RORγ$^{-/-}$ mice but was readily detected in wild-type controls (**Fig. 7a**). In contrast, V_α6, near the 3′ end of the V_α locus, was used in both the mutant mice and littermate controls (**Fig. 7a**). Usage of V_α19 was restored by Bcl-x_L transgene expression in RORγ$^{-/-}$ mice (**Fig. 7a**), which indicated that the lack of V_α19 usage in RORγ$^{-/-}$ mice is due to a shortened DP lifespan. Thus, the lack of 3′ J_α rearrangement

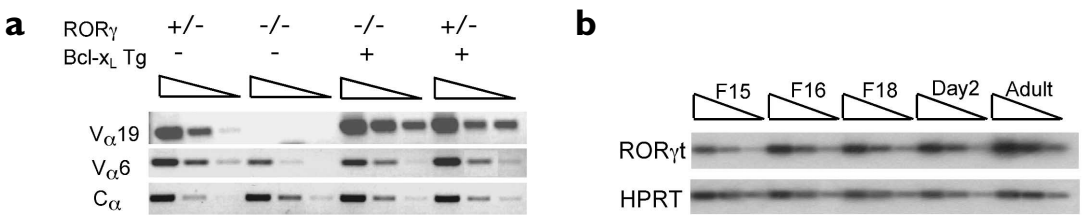


Figure 7. Impaired 5′ V_α usage in RORγ$^{-/-}$ mice. (**a**) Total RNA of thymocytes from RORγ$^{-/-}$, RORγ$^{+/-}$, RORγ$^{-/-}$ Bcl-x_L Tg and Bcl-x_L Tg mice was subjected to semi-quantitative RT-PCR analysis of the expression of V_α19 and V_α6. Samples were serially diluted at 1:5. PCR with C_α primers was used for template normalization. PCR products were probed with an internal C_α oligonucleotide. (**b**) Expression of RORγt in developing thymocytes. Total RNA from thymi of day 15–18 fetal (F15, F16 and F18), neonatal (Day 2) or adult (Adult) mice was subjected to RT-PCR analysis of the expression of RORγt. Samples were serially diluted at 1:5. PCR products were detected with a 400-bp RORγt probe.

in RORγ$^{-/-}$ mice is accompanied by a lack of 5′ V_α rearrangement. As both defects can be corrected by Bcl-x_L transgene expression, the data give strong support to a bidirectional and coordinated model for TCRα recombination.

Expression of RORγt in developing thymocytes

Several reports showed that J_α usage in fetal and neonatal T cells is highly restricted to the 5′ end of the J_α locus[2,4]. This pattern of J_α usage is similar to that in RORγ$^{-/-}$ mice and raises the possibility that RORγt expression may be developmentally regulated. To test this, we examined the expression of RORγt in developing thymocytes. RORγt was readily detectable in the thymi of fetal and neonatal mice (**Fig. 7b**). The slightly reduced amount of RORγt expression in day 15 fetal thymi is likely due to a limited number of DP thymocytes at this stage. This result suggests that factors other than RORγt expression may limit J_α usage in neonatal thymocytes.

Discussion

Our data allow us to draw three key conclusions regarding the regulation of thymocyte development and TCR repertoire formation. First, we show that the orphan nuclear receptor isoforms RORγt and RORγ promote efficient thymocyte maturation from the ISP to the DP stage of thymocyte development. Second, we show that the lifespan of DP thymocytes determines how far TCRα rearrangement proceeds along the J_α locus. Thus, the shortened lifespan of RORγ$^{-/-}$ DP thymocytes results in impaired rearrangement to 3′ J_α segments. In contrast, the prolonged lifespan of DP thymocytes in Bcl-x_L Tg mice skews J_α rearrangements to the extreme 3′ end of the J_α locus. Third, we show that the peripheral TCRα repertoire in Bcl-x_L Tg mice is skewed towards usage of more 3′ J_α segments as well, which demonstrates that the survival window of DP cells is a key factor that regulates formation of the peripheral TCRα repertoire.

Our studies show that a key function of RORγt and/or RORγ is to promote the ISP to DP step of thymocyte maturation. The molecular events that control the ISP to DP transition are poorly defined. Two other transcription factors—TCF-1 (T cell factor 1) and HEB (Hela E-box–binding protein)—regulate this step, as mice that are deficient in either factor show a delay in the ISP to DP transition, similar to that reported here[33,34]. The impaired ISP to DP transition in RORγ$^{-/-}$ thymocytes is not due to the influence of RORγt and/or RORγ on Bcl-x_L expression. This is because an increased ISP thymocyte population is still observed in RORγ$^{-/-}$ Bcl-x_L Tg mice, even though the massive apoptosis of DP cells is corrected[21]. In addition, a similar delay is not observed in Bcl-$x_L^{-/-}$ mice[35,36]. Because Bcl-x_L Tg expression rescues 3′ J_α usage in RORγ$^{-/-}$ mice but does not rescue the ISP to DP transition, the J_α phenotype of RORγ$^{-/-}$ mice is independent of the ISP maturation defect. RORγ$^{-/-}$ mice may prove useful in future studies that aim to dissect the molecular events that regulate this transition.

DP thymocytes meet one of three fates in the thymus[37]. A small fraction of DP thymocytes express TCRs with intermediate affinity for self-peptide–major histocompatibility complex (self-pMHC) and are therefore positively selected and exported to the periphery. DP thymocytes that express a TCR with too high an affinity for self-pMHC are deleted *via* negative selection. However, the vast majority of DP thymocytes undergo "death by neglect" because they fail to express a TCR or express a useless TCR that is of insufficient affinity to interact with self-pMHC ligands. The ability of DP thymocytes to undergo multiple rounds of TCRα rearrangement is thought to maximize the chance of forming a useful TCR[6]. Generation of a TCR that is suitable for positive selection will terminate the process of V_α to J_α rearrangement by down-regulating RAG gene expression[12–14]. However, if such a TCR is not generated, secondary V_α to J_α rearrangements will proceed down the J_α locus towards the 3′ end. As such, it might be assumed that DP thymocytes undergo programmed cell death after having exhausted the entire J_α array and all opportunities to create a selectable TCR. Our results indicate that this is not the case: programmed cell death actually limits the process of TCRα rearrangement in a substantial portion of developing thymocytes and prevents these cells from testing J_α segments at the 3′ end of the J_α array. Therefore, an increase in DP thymocyte lifespan is expected to increase the number of positively selected T cells and, hence, the efficiency of positive selection. We found that an increase in DP lifespan results in a peripheral repertoire with an unusual 3′ bias in terms of J_α usage. Thus, the programmed cell death of DP cells should be seen not simply as a consequence of failed selection, but as an important regulator of the TCRα repertoire, with the DP lifespan normally set to yield evenly distributed J_α usage in the periphery.

Our conclusions assume that the only relevant effect of Bcl-x_L expression in DP thymocytes is on their survival. Bcl-x_L is specifically expressed in high amounts in DP thymocytes and it is well documented that it promotes their survival[35,36]. Because Bcl-x_L transgene expression does not perturb negative selection[38,39], it seems likely that it acts to prolong the survival of DP thymocytes that would otherwise die of neglect. Bcl-x_L is documented to have additional effects on thymocyte development because over-expression of Bcl-x_L in DP thymocytes promotes the formation of CD8$^+$ SP thymocytes, even in mice that transgenically express an MHC class II–restricted TCR[39]. Similarly, Bcl-2 over-expression promotes CD8$^+$ T cell maturation in MHC class I$^{-/-}$ mice[40]. However, in neither case were these CD8$^+$ T cells exported to the periphery, which indicates that Bcl-x_L or Bcl-2 expression cannot fully substitute for positive selection signals and would not perturb the peripheral TCR repertoire[39,40].

Our results indicate that the progression of secondary rearrangements across the J_α locus is critically sensitive to DP thymocyte lifespan: the average 3-day lifespan of wild-type DP thymocytes is insufficient for the majority of rearrangement events to progress to the 3′ end

of the J_α array. Why might this be the case? It is believed that, due to the activity of the TEA promoter and perhaps additional promoters associated with 5′ J_α segments, primary TCRα rearrangements are focused to the 5′ end of the J_α locus[16,41]. We consider that secondary rearrangements could proceed down the locus either *via* large steps that might bypass many J_α segments and test only a minority ("express service") or in small steps that might bypass few J_α segments and test a large number ("local service"). We suggest that acute sensitivity to DP lifespan implies that transit of the J_α locus is a slow process with "local" rather than "express" service.

What factors might enforce "local service" across the J_α locus? Chromatin structure plays a critical role in developmental regulation of V(D)J recombination[42]. J_α chromatin becomes hyperacetylated in DP thymocytes, which correlates with the ability of DP thymocytes to initiate V_α to J_α rearrangement[18]. By the criterion of histone acetylation alone, the entire J_α locus would be considered accessible in DP thymocytes from RAG-2$^{-/-}$ TCRβ Tg mice, and the unrearranged 3′ portion of the J_α locus would also be considered accessible in DP thymocytes from RORγ$^{-/-}$ mice. If histone acetylation alone determined accessibility for V(D)J recombination, it would be difficult to explain the focusing of initial rearrangements to the 5′ end of the J_α locus, difficult to prevent "express service" secondary rearrangements from proceeding rapidly across the J_α locus and difficult to explain the lack of 3′ J_α usage in RORγ$^{-/-}$ mice. *In vitro* studies have shown that histone acetylation and ATP-dependent chromatin remodeling complexes work together to provide accessibility to RAG proteins[43]. Also, *in vivo* studies of a TCRβ minilocus have indicated that histone acetylation of D_β and J_β segments alone is insufficient for recombinational accessibility. Promoter proximity to D_β is critical, suggesting that promoters contribute additional required remodeling activities that act only locally (M. L. Sikes, R. Tripathi, M. S. Krangel and E. M. Oltz, unpublished data).

These considerations could explain "local service" across the J_α locus in the following way. Activation of Eα and its interaction with the TEA and other 5′ promoters in the J_α locus stimulates acetylation of histone residues across the locus and stimulates additional remodeling events in the vicinity of the 5′ promoters. These changes allow RAG access to 5′ J_α segments and promote their rearrangement. Primary TCRα rearrangement can then replace the endogenous 5′ promoters with a newly introduced V_α promoter. This promoter remodels chromatin for a relatively short additional distance downstream (perhaps a few J_α segments), stimulating the next round of rearrangement to the J_α segments immediately downstream of the primary rearrangement. This process would then be repeated until it is terminated by either positive selection or cell death. An alternative to explain the tracking of rearrangements down the J_α locus is that discrete windows are sequentially opened during development through the activation of developmentally staged germline regulatory elements such as promoters. This seems less likely because germline promoter activity seems to be restricted to the 5′ end of the locus[41]. In addition, this scenario has been argued against on the basis of uncoordinated J_α rearrangements on the two alleles of TEA$^{+/-}$ mice[9].

Thus, the J_α locus may have evolved in a manner that is not only permissive for secondary rearrangements, but that also enforces "local service" down the J_α array. "Local service" is slow and hence is limited by DP thymocyte lifespan. Nevertheless, it is presumably the most effective strategy, as it provides thymocytes with maximal opportunity to generate a useful TCR.

Methods

Mice. RORγ$^{-/-}$ mice[21], which were backcrossed to a C57BL/6 background for five generations, were housed in a specific pathogen–free facility at the Duke University Vivarium. Heterozygous mice were bred to generate homozygous mutant mice and control heterozygous littermates. RAG-2$^{-/-}$ TCRβ Tg[30] and Eα$^{-/-}$[17] mice were similarly housed. Fetal thymi were dissected from pregnant C57BL/6 mice (Jackson Laboratory, Bar Harbor, MI) with the day that the plug was detected counted as day 0. Animal usage was carried out according to protocols approved by the Duke University Institutional Animal Care and Use Committee.

Flow cytometric analysis and sorting. The following antibodies were from either BD PharMingen (San Diego, CA) or eBioscience (San Diego, CA): phycoerythrin (PE)–anti-CD3 (145-2C11), fluorescein isothiocyanate (FITC)– or cychrome–anti-CD4 (GK1.5), PE–anti-CD5 (53-7.3), PE– or cychrome–anti-CD8α (53-6.7), PE–anti-CD24 (30-F1) and FITC-anti–H-2K^b (AF6-88.5). Thymocytes or splenocytes from mutant or control mice were stained with antibodies on ice for 30 min, washed with PBS that contained 0.5% bovine serum albumin (BSA) and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and CellQuest software. Events (1×10^4–2×10^4) were collected for each sample. DP thymocytes were obtained at >98% purity by staining with FITC–anti-CD4 and PE–anti-CD8 followed by cell sorting. For isolation of ISP cells, thymocytes from normal C57BL/6 mice were incubated with an excess amount of biotin–anti-CD4 (RM4-5); this was followed by removal of DP and CD4$^+$ SP cells with streptavidin-conjugated Dynabeads (Dynal ASA, Oslo, Norway). The remaining cell populations were stained with FITC–anti-CD8, PE–anti-CD4 (GK1.5), PE–anti-CD3 and PE-streptavidin. CD8$^+$CD4$^-$CD3$^-$ ISP cells were sorted to >98% purity by FACS.

Isolation of DNA and RNA, RT-PCR and northern blot analysis. RNA was isolated from total tissue or sorted DP thymocytes with TRIzol (Gibco-BRL, Grand Island, NY) according to the manufacturer's instructions. Genomic DNA was isolated from the interface after the RNA-containing aqueous phase was removed. cDNA was synthesized with Superscript II reverse transcriptase (Gibco-BRL) with oligo dT primers. PCR conditions for amplifying V_α to C_α products were 94 °C for 3 min and then 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, followed by a 10 min extension at 72 °C. Serially diluted cDNA (1:5) was analyzed for the expression of TCRα, Bcl-x_L, RAG-1, RAG-2, RORγt and hypoxanthine phosphoribosyltransferase (HPRT) by semi-quantitative PCR as follows: 94 °C for 2 min and then 30 cycles of 94 °C for 30 s, 55–58 °C for 30 s and 72 °C for 1 min. PCR products were probed with cDNA probes of their encoding regions. Total RNA (10 μg) from thymocytes were analyzed by northern blotting with a standard protocol[44] and probes to the C_α portion of TCRα and glyceraldehyde-3-phosphate dehydrogenase (GAPDH**).**

Long-range PCR. Genomic DNA from sorted DP thymocytes (100 ng) was subjected to long-range PCR with the use of primers as described[26] and a GC Genomic PCR kit (Clontech, Palo Alto, CA). PCR conditions were as follows: 95 °C for 2 min and then 40 cycles of 94 °C for 30 s, 62 °C for 30 s and 68 °C for 5 min, followed by an extension at 68 °C for 5 min. PCR products were separated on a 1% agarose gel and transferred to a Duralon-UV membrane (Stratagene, La Jolla, CA) for Southern blot analysis.

Southern blot analysis. Membranes carrying V_α-C_α RT-PCR products or PCR products of genomic DNA were prehybridized in 6× SSC (saline sodium citrate), 5× Denharts and 0.5% SDS for 1–2 h at 42 °C and incubated with end-labeled J_α oligonucleotide probes for an additional 4–5 h. A control C_α exon I oligonucleotide probe was used for normalization. Membranes were washed with 6× SSC twice for 10 min each and 2× SSC and 0.1% SDS once for 15 min at room temperature. For sequential hybridization, blots were stripped by boiling the membranes three times for 5 min. Each was confirmed by exposing the membranes to film. Oligonucleotide sequence for the V_α19 forward primer: 5′–TCTGACAGAGCTCCAGATCAA–3′; V_α6 forward primer: 5′–GATCCTGACTCATG TCAGCC–3′ . J_α and C_α oligonucleotides were as described[16,26]. J_α nomenclature is as described[45].

ChIP. Ten-million sorted CD4$^+$CD8$^+$ thymocytes from RORγ$^{-/-}$ mice and 40×10^6 unfractionated thymocytes from RAG-2$^{-/-}$ TCRβ Tg and Eα$^{-/-}$ mice were cross-linked with formaldehyde and processed for ChIP essentially as described[46] (modifications suggested by G. Beresford, Emory University, were introduced). Sonicated chromatin was precleared with salmon sperm DNA–protein A agarose slurry (Upstate Biotechnology, Lake Placid, NY) and was immunoprecipitated with either rabbit polyclonal anti–diacetylated histone H3 (Upstate Biotechnology) or control immunoglobulin G (Sigma, St. Louis, MO) followed by salmon sperm DNA–protein A agarose slurry. The unbound fraction of the control immunoprecipitation was saved and used as input DNA. Slurry–immune complexes were washed twice with immunoprecipitation dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl at pH 8.1 and 167 mM NaCl), twice with 500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA and 20 mM Tris-HCl at pH 8.1, twice with 100 mM Tris-HCl (at pH 8.1), 500 mM LiCl, 1% NP-40, 1% deoxycholic acid and twice with 10 mM Tris at pH 8.0 and 1 mM EDTA. DNA samples prepared from the antibody-bound and input fractions were dissolved in equal volumes of 10 mM Tris-HCl (pH 8.0). Bound fractions were used in PCR analyses either undiluted or at dilutions of 1/5 and 1/25; input fractions were used at 1/200, 1/1000 and 1/5000. Amplification (20 s at 92 °C, 30 s at 56 °C or 60 °C and 20 s at 72 °C) was for 25–30 cycles. PCR products (120–140 bp) were electrophoresed through 1.5% agarose, transferred to nylon and detected by hybridization with oligonucleotide probes radiolabeled with T4 polynucleotide kinase. Hybridization signals were analyzed with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Raw acetylation values were derived by determining the displacement between titration curves for the anti–diacetylated H3 immunoprecipitate and input. Normalized acetylation values were determined relative to the amount of actin acetylation in each chromatin sample. Oligonucleotides used were as follows.

Oct-2: forward (Oct5)[18], reverse (Oct6)[18] and probe (Oct4) 5′–TGAAGGTGCGAG-CAAACTGT–3′. Actin: forward 5′–GTCATTCCAAGTATCCATGAAA–3′, reverse 5′–AAGCAATGCTGTCACCTTCC–3′ and probe 5′–AAAGCCACCCCCACTCCTA–3′. $J_{\alpha}30$: forward 5′–TAACAGCATTGTGTTGGACATCCC–3′, reverse 5′–TCCCTCCTTGT-TATTTTGCCTG–3′, and probe 5′–TCACTCTCAGTGTCAAATGAGGTCAATAAG–3′. $J_{\alpha}15$: forward 5′–GCATACACCATGAGTTTGAATGAG–3′, reverse 5′–AGCTATCAGG TACTTACTGGGG–3′ and probe 5′–GGAGGCAGAGCTCTGATATTT–3′. $J_{\alpha}7$: forward 5′–TCCTCCGTGAGAGGGTAGAG–3′, reverse 5′–AGGACATAGCTCACTTACTTGG–3′ and probe 5′–GGACTACAGCAACAA CAGACTTACTTTGGGGA–3′.

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Competing interests statement

The authors declare that they have no competing financial interests.

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