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Regulation of *Tcra* gene assembly by a complex hierarchy of germline J_α promoters

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Abstract

Tcra gene assembly is characterized by an orderly progression of primary and secondary V_α to J_α recombination events across the J_α array, but the targeting mechanisms responsible for this progression are largely unknown. Previous studies revealed that the TEA promoter plays an important role in targeting primary *Tcra* rearrangements. We show that TEA and a novel promoter associated with J_α49 target primary recombination to discrete sets of C_α-distal J_α segments and together direct nearly all normal primary recombination events. Further, we show that TEA promoter deletion activates previously suppressed downstream promoters and stimulates primary rearrangement to centrally located J_α segments. Central promoter derepression also occurs following primary rearrangement, thereby providing a mechanism to target secondary recombination events.

The development of T lymphocytes bearing an αβ T cell receptor (TCR) depends on the somatic assembly of variable (V), diversity (D) and joining (J) gene segments by the process of V(D)J recombination[1]. *Tcrb* gene rearrangements occur first, in the $CD4^-CD8^-$ double negative (DN) subset of thymocytes. Production of a functional TCRβ protein then promotes differentiation to the $CD4^+CD8^+$ double positive (DP) stage, during which *Tcra* gene rearrangements occur. Following expression of a cell surface αβ TCR, DP thymocytes are tested by positive selection to identify those with useful TCRs[2]. Only a small fraction of DP thymocytes are ultimately selected for further maturation.

Tcra rearrangement has several unique features that are thought to increase the likelihood of positive selection[2,3]. First, V_α to J_α rearrangement occurs on both alleles without allelic exclusion. Second, initial, or primary rearrangements, are targeted to J_α segments at C_α-distal, or 5′ end, of the 70 kb J_α array (polarity is denoted in reference to the sense strand throughout). If primary rearrangement fails to produce a selectable TCR, DP thymocytes may then undergo multiple additional rounds of secondary rearrangement that involve progressively more 5′ V_α gene segments and progressively more 3′ J_α gene segments. Moreover, the 5′ to 3′ progression along the J_α array is nearly synchronous on two *Tcra* alleles. *Tcra* rearrangement is ultimately terminated by positive selection, which silences recombinase expression, or by cell death resulting from a lack of positive selection. Thus, secondary V_α to J_α rearrangement provides thymocytes multiple opportunities for positive selection, and is critical for the production of a robust and diverse TCRα repertoire[4,5].

The mechanisms that direct primary *Tcra* rearrangement are only partially understood. The *Tcra* enhancer (E_α), situated 3′ of C_α, controls the entire J_α array and is required for all V_α to J_α recombination events[6]. The T early α (TEA) promoter, located at the 5′ end of the J_α array, acts more locally, and controls usage of the most 5' J_α segments (J_α61-J_α53)[7]. However, TEA-deficient mice have quantitatively normal J_α usage downstream of this region, suggesting the

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presence of one or more additional *cis*-acting elements with the potential to target primary rearrangements. We previously analyzed the distribution of J_α usage in the short-lived DP thymocytes of mice lacking transcription factor RORγ(*Rorc*$^{-/-}$)[5]. J_α usage was restricted to the 5′ portion of the J_α array, as would be expected if thymocytes were limited to primary rearrangements. Moreover, the detected rearrangements resolved into two discrete clusters, one just downstream of TEA and one spanning J_α50 to J_α45. The former is presumed to reflect primary rearrangements targeted by the TEA promoter. The latter appears to identify a site of primary targeting that could account for the residual rearrangements in TEA-deficient thymocytes. However, to date, no known regulatory elements have been mapped to this region.

Even less is understood about the targeting of secondary *Tcra* rearrangements. An orderly 5′ to 3′ progression of rearrangements across the J_α array is implied by allelic synchrony in J_α usage. Two types of models have been proposed, both relying on the ability of promoters to provide local access to the recombinase[5,7,8]. The developmental windows model envisions a set of embedded germline J_α promoters that are successively activated according to an intrinsic developmental program during DP thymocyte development. An alternative model envisions a V_α promoter-driven chain reaction, in which, following primary rearrangement, successively introduced V_α promoters would target subsequent rounds of secondary rearrangement to progressively more 3′ sets of J_α segments.

To better understand the molecular basis for the ordered propagation of rearrangement events across the J_α array, we searched this portion of the *Tcra* locus for novel promoter elements. We identified a strong promoter element situated just upstream of J_α49, and tested its functional relevance by deleting it, either alone or in combination with the TEA promoter, using homologous recombination strategies. Our results indicate that the two promoters target discrete sets of 5′ J_α segments for primary rearrangement, and together account for almost all normal primary J_α recombination events. In addition, we found that 5′ promoter deletion leads to the activation of previously suppressed downstream promoters, which can efficiently target rearrangements into the central portion of the J_α array. Central promoter derepression also occurs following primary rearrangement. This provides a previously unappreciated mechanism for the targeting of secondary V_α to J_α recombination events.

RESULTS

Identification of a germline promoter upstream of J_α49

We searched for the presence of promoter elements that could account for the recovery of J_α rearrangements downstream of J_α53 in TEA-deficient mice[7]. As one approach, DP thymocytes from *Rag2*$^{-/-}$ mice carrying a functional *Tcrb* transgene (Rxβ) were analyzed for novel DNase I hypersensitive sites within the J_α array (**Fig. 1a,b**). These mice provide a pure source of DP thymocytes that retain both a germline J_α array and all associated promoter elements. Based on Southern hybridization using a J_α50 probe, DNase I digestion reduced a 12 kb *Spe*I fragment to a 5.5 kb species. This mapped a strong hypersensitive site just upstream of the J_α49 gene segment. In the second approach, a C_α primer was used in 5' rapid amplification of cDNA ends (RACE) to amplify full-length germline transcripts that span the J_α array (**Fig. 1a,c**). Such transcripts are typically spliced from the most 5′ splice donor to C_α. Two major species of 1.3 kb and 0.6 kb were amplified from cDNA of Rxβ DP thymocytes (**Fig. 1c, inset**). These species were cloned and sequenced. The 1.3 kb products were found to reflect initiation at the TEA promoter at the extreme 5′ end of the J_α array, with the TEA exon spliced to C_α (data not shown). The 0.6 kb products reflected initiation at sites associated with J_α segments (J_α58-J_α44), with splicing from J_α to C_α. The predominant germline J_α transcripts included J_α49 and initiated at several sites clustered within the J_α49 recombination signal sequence (**Fig. 1c,d**). To test for J_α49 promoter function *in vitro*, a 900 bp fragment spanning the J_α49 transcription start site was cloned upstream of the luciferase gene in a reporter plasmid that contained or

lacked E_α, and was tested for activity following transfection into Jurkat T cells (**Fig. 1e**). When introduced into LUC-E_α, the promoter fragment stimulated luciferase activity by about 200-fold. However, promoter activity was minimal in reporter substrates lacking E_α. All activity of the 900 bp fragment was contained within a 5′ truncated fragment of 425 bp (data not shown). The position of the J_α49 promoter corresponds to the region of the J_α array in which J_α usage increases to wild-type frequency in TEA-deficient mice[7] and to the cluster of primary J_α recombination events observed in the short-lived DP thymocytes of *Rorc*$^{-/-}$ mice[5]. In contrast, promoter activity associated with the J_α58, J_α57, and J_α56 gene segments (**Fig. 1c**) localized within the TEA-dependent zone. Therefore, we focused on the J_α49 promoter as a putative targeting element for primary V_α to J_α recombination.

T cell development in promoter-deleted mice

The J_α49 and TEA promoters were individually deleted from embryonic stem (ES) cells derived from 129:C57BL/6 (B6) heterozygous mice using a Cre-*lox*P homologous recombination strategy (**Fig. 2a,b**). Chimeric mice carrying a *lox*P-flanked *neo*^{r} cassette in place of TEA were bred to transgenic mice mice expressing Cre recombinase to delete the cassette, creating a TEA-deficient allele (ΔTEA). The *lox*P-flanked *neo*^{r} cassette was removed from J_α49 promoter-targeted ES cells by transient expression of Cre recombinase *in vitro* to create a J_α49-deficient allele (ΔJ49). These cells were then retargeted to delete the TEA promoter as well. The use of heterozygous ES cells insured that the two homologous recombination events would occur on the same (129) allele. Cre-mediated recombination then yielded two different alleles, one with simple deletions of both promoters (ΔTEAΔJ49) and a second with a 16 kb deletion encompassing the entire region between TEA and J_α49 (Δ5′) (**Fig. 2c**). The behavior of all four targeted alleles was subsequently analyzed in homozygous mice (ΔTEA, ΔJ49, ΔTEAΔJ49, and Δ5′).

To test for developmental defects in the various strains, CD4, CD8 and TCRβ expression was analyzed in thymocytes and splenocytes of 2-5 week old mice by flow cytometry. No differences were detected in the absolute numbers and percentages of DN, DP, and single positive thymocytes and splenic T lymphocytes as compared to age matched littermate controls (data not shown). Similarly TCRβ expression on thymocytes and splenocytes was indistinguishable from littermate controls. Thus, there were no gross deficiencies in αβ TCR expression or αβ T cell development in promoter-deleted mice.

The J_α49 promoter controls primary rearrangement

To study the effect of promoter deletions on primary targeting to the J_α locus, ΔJ49 and ΔTEAΔJ49 alleles were bred onto the *Rorc*$^{-/-}$ background. V_α8 and C_α primers were used to amplify cDNA prepared from *Rorc*$^{-/-}$, ΔJ49*Rorc*$^{-/-}$, and ΔTEAΔJ49*Rorc*$^{-/-}$ thymocytes, and polymerase chain reaction (PCR) products were cloned and sequenced to identify the distribution of J_α segments used (**Fig. 3**). Because the V_α8 primer detects multiple V_α8 family members that are dispersed across the V_α region, V_α8 rearrangements are relatively unbiased with respect to J_α usage[5]. Consistent with previous results[5], two clusters of J_α usage were detected in *Rorc*$^{-/-}$ thymocytes, one encompassing J_α58 and J_α57, and a second encompassing J_α50 to J_α45. The second cluster was absent in ΔJ49*Rorc*$^{-/-}$ mice, indicating that the J_α49 promoter is required for primary rearrangements to the region spanning from J_α50 to J_α45. Both clusters were absent in ΔTEAΔJ49*Rorc*$^{-/-}$ mice, indicating that the TEA promoter is required for primary rearrangements to the most 5′ J_α segments, and that the two promoters together are required for almost all normal primary targeting events into the J_α array. Without these promoters, J_α usage in short-lived DP thymocytes was much more broadly distributed, although nearly 60% of rearrangements involved J_α42 to J_α26.

Altered T cell repertoire in promoter-deleted mice

To analyze the effects of altered primary rearrangement on the J_α repertoire, cDNA was prepared from wild-type, ΔTEA, ΔJ49, ΔTEAΔJ49 and Δ5' thymocytes, and was amplified using $V_\alpha 8$ and C_α primers. PCR products were then fractionated on agarose gels and transferred to nylon filters, and J_α usage was evaluated by hybridization with J_α-specific oligonucleotide probes (**Fig. 4**). In comparison to wild-type thymocytes, ΔJ49 thymocytes displayed an overrepresentation of J_α segments from $J_\alpha 58$ to $J_\alpha 50$, with normal to slight underrepresentation of J_α usage further downstream. We interpret elevated usage of the most 5′ J_α segments to reflect an increase in primary targeting to the TEA-dependent zone in the absence of the $J_\alpha 49$ promoter. The relatively normal usage of J_α segments across the $J_\alpha 49$ promoter-dependent zone suggests that in the absence of primary targeting by the $J_\alpha 49$ promoter, these J_α segments can still be used efficiently as a consequence of secondary rearrangements that follow primary rearrangements targeted by the TEA promoter.

ΔTEA mice revealed a profile of J_α usage similar to that reported previously[7]. By comparison, ΔTEAΔJ49 mice were more substantially impaired, with J_α usage further depressed by an average of 25% between $J_\alpha 50$ and $J_\alpha 38$. Δ5′ mice revealed a nearly identical profile, except that $J_\alpha 48$ usage was suppressed as compared to ΔTEAΔJ49 mice. That $J_\alpha 49$ promoter deletion impairs usage of $J_\alpha 50$ to $J_\alpha 38$ on a ΔTEA but not a TEA^+ background must reflect the elimination of secondary rearrangements that follow primary targeting by TEA. Thus, although the TEA and $J_\alpha 49$ promoters target primary rearrangements in nonredundant fashion, they have partially redundant influences on J_α usage as a consequence of secondary rearrangements. Because $J_\alpha 52$ and $J_\alpha 50$ are still used inefficiently on ΔTEAΔJ49 alleles, there may be weak targeting elements in the TEA to $J_\alpha 49$ interval, other than the TEA and $J_\alpha 49$ promoters. These elements must also account for low frequency $J_\alpha 48$ usage on ΔTEAΔJ49 alleles, given that $J_\alpha 48$ usage is minimal on Δ5′ alleles. Downstream of $J_\alpha 48$, there was a gradual increase in J_α usage in both ΔTEAΔJ49 and Δ5′ mice, first to physiologic and then to superphysiologic amounts. This result indicates the presence of additional *cis*-acting elements that can target primary rearrangements to this region, consistent with the modified profile of primary rearrangements in ΔTEAΔJ49*Rorc*$^{-/-}$ mice (**Fig 3**).

The preceding experiments monitored J_α usage relative to C_α usage in each mouse strain, but did not measure the absolute magnitude of rearrangement across the central portion of the J_α array. For this purpose, we quantitatively analyzed restriction digests of thymocyte genomic DNA by Southern hybridization. We generated heterozygous mice carrying a ΔTEAΔJ49 129 allele and a wild-type B6 allele, and exploited restriction fragment length polymorphisms to simultaneously examine rearrangement of the two alleles in individual DNA samples (**Fig 5a**). The extent of recombination events involving a set of J_α gene segments was evaluated by measuring loss of the germline (unrearranged) restriction fragment on which they are located. Previous studies of wild-type alleles showed rearrangement to be extensive at the 5′ end of the J_α array, and to diminish gradually from 5′ to 3′[7,9,10]. Consistent with this, we observed that on the B6 allele, retention of the germline $J_\alpha 61$-$J_\alpha 56$ fragment (Region 1) was only 29%, whereas retention of the germline $J_\alpha 50$-$J_\alpha 43$ fragment (Region 2) was 44% and retention of the germline $J_\alpha 42$-$J_\alpha 37$ fragment was 58% (**Fig. 5a,b**). Previous analysis of TEA-deficient alleles revealed almost complete retention of the germline signal for $J_\alpha 61$-$J_\alpha 53$, but only 20-25% retention immediately downstream ($J_\alpha 53$-$J_\alpha 39$)[7]. This indicates that recombination events involving 5′ J_α segments are rare, whereas J_α segments immediately 3′ rearrange at high frequency. Germline signals for the 5′ J_α segments persist despite extensive rearrangement of more 3′ J_α segments because the unrearranged 5′ J_α segments are excised onto extrachromosomal circles that are stably maintained in DP thymocytes. Consistent with repertoire analysis (**Fig. 4**), rearrangement on the ΔTEAΔJ49 allele was more substantially impaired than on a ΔTEA allele, since the retention of germline signal was 95% for $J_\alpha 61$-$J_\alpha 56$

(Region 1) and 74% for $J_\alpha 48$-$J_\alpha 44$ (Region 2). Nevertheless, rearrangement on the ΔTEAΔJ49 allele was equivalent to B6 for $J_\alpha 43$-$J_\alpha 37$ (Region 3). That recombination efficiency of the mutant allele was similar to wild-type in Region 3 implies that with the TEA and $J_\alpha 49$ promoters missing, downstream elements can efficiently target primary rearrangements to 3′ J_α segments, and thereby promote a physiological amount of V_α to J_α recombination.

J_α chromatin structure in promoter-deleted mice

Chromatin that is accessible for V(D)J recombination typically displays increased acetylation of histones H3 and H4[11]. Therefore, to understand the targeting and recovery of rearrangements across the central portion of the J_α array, we analyzed the effects of promoter deletion on the acetylation state of J_α histones. For this purpose, ΔJ49 and ΔTEAΔJ49 alleles were bred onto a Rxβ background, and mononucleosomes were prepared from Rxβ thymocytes, ΔJ49Rxβ thymocytes, ΔTEAΔJ49Rxβ thymocytes, and as a control, from T cell-deficient splenocytes isolated from *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice. Acetylated mononucleosomes were immunoprecipitated using antibodies specific for diacetylated histone H3 and tetraacetylated histone H4, and real-time PCR was used to quantify acetylation at sites along the J_α array (**Fig. 6**). In Rxβ thymocytes acetylation was found to be high across the most 5′ J_α gene segments and to decrease gradually towards the 3′ portion of the array. In contrast, the entire array J_α array was hypoacetylated in *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ splenocytes. As compared to the histone acetylation found in Rxβ thymocytes, H3 and H4 acetylation in ΔJ49Rxβ thymocytes was normal at the TEA exon but was partially reduced from $J_\alpha 61$ through $J_\alpha 45$. In contrast, ΔTEAΔJ49Rxβ thymocytes revealed a completely different profile. Relative to ΔJ49Rxβ mice, histone acetylation was substantially reduced from the TEA exon through $J_\alpha 48$, but was substantially increased at multiple sites further 3′. Previous work had shown that TEA deletion reduced *Tcra* locus acetylation from TEA through $J_\alpha 53$[12]. The current data imply that the TEA and $J_\alpha 49$ promoters collaborate to maintain a normal amount of histone acetylation through $J_\alpha 48$. More importantly, the detection of normal to elevated acetylation downstream of $J_\alpha 48$ is consistent with the presence of an additional set of *cis*-acting elements that could target primary rearrangement (**Fig. 3**) and an increase to a physiologically normal amount of J_α usage (**Figs. 4** and **5**) across the central portion of the J_α array.

Activation of downstream promoters

The analysis of germline transcripts in Rxβ DP thymocytes identified a low frequency of germline transcripts initiating at $J_\alpha 46$, $J_\alpha 45$, and $J_\alpha 44$ (**Fig. 1c**). To assess the activity of centrally located promoter elements following 5′ promoter deletion, germline C_α transcripts in DP thymocytes of ΔTEAΔJ49Rxβ mice were analyzed by 5' RACE (**Fig. 7a**). Sequence analysis identified germline transcripts initiating upstream of and within $J_\alpha 47$, $J_\alpha 45$, $J_\alpha 44$, $J_\alpha 42$, $J_\alpha 40$, $J_\alpha 37$ and $J_\alpha 7$, as well as just downstream of $J_\alpha 37$. No germline transcripts were identified at $J_\alpha 58$ and $J_\alpha 57$, even though these transcripts were well represented in Rxβ DP thymocytes, and the associated promoters should still have been present. A similar profile of transcripts had previously been described in TEA-deficient DP thymocytes[13]. However in neither case was it clear whether the transcripts associated with centrally located J_α segments reflected residual transcription that could be more readily detected once 5′ promoters were deleted, or rather, reflected *bona fide* increases in promoter activity.

To address this issue, we analyzed the abundance of selected germline $J_\alpha C_\alpha$ transcripts by semiquantitative RT-PCR (**Fig. 7b**). This analysis allows us to compare how a specific germline transcript varies in abundance among different genotypes. However as presented, it does not provide direct information about the relative abundance of different germline transcripts. The quantity of total germline C_α transcripts was similar in Rxβ and ΔJ49Rxβ DP thymocytes but was reduced by at least 90% in ΔTEAΔJ49Rxβ DP thymocytes. This result is consistent with previous analysis of TEA-deficient mice, which revealed that the majority of

germline C_α-containing transcripts depend upon the TEA promoter[7]. Consistent with the 5' RACE analysis, transcripts initiating from $J_\alpha 58$ were undetectable in ΔTEAΔJ49Rxβ DP thymocytes. Even more striking, transcripts initiating from $J_\alpha 47$, $J_\alpha 42$, and $J_\alpha 37$ were increased about 10-fold in ΔTEAΔJ49Rxβ as compared to Rxβ thymocytes. Because the pattern of transcription on ΔTEAΔJ49 alleles mirrored the pattern previously documented for TEA-deficient alleles[7], the inhibition of $J_\alpha 58$ and induction of $J_\alpha 47$, $J_\alpha 42$, and $J_\alpha 37$ transcripts in these thymocytes must reflect TEA promoter deletion per se. This conclusion was strengthened by the finding that $J_\alpha 49$ promoter deletion resulted in downregulation rather than upregulation of $J_\alpha 47$ and $J_\alpha 42$ transcripts. Moreover, because of this downregulation, the magnitude of induction of these transcripts as a consequence of TEA promoter deletion was even greater than the 10-fold increase noted in comparison of ΔTEAΔJ49Rxβ to Rxβ thymocytes. Thus, deletion of the TEA promoter dramatically downregulates transcription initiating at the 5′ end of the J_α array, but reciprocally upregulates transcription initiating within the central portion of the array. This refocusing of promoter activity likely accounts for the retargeting of primary J_α rearrangements in 5′ promoter-deleted mice.

Were central promoter induction to occur when the TEA promoter is deleted by primary rearrangement, it would provide a mechanism for the targeting of secondary rearrangements into the central portion of the J_α array. To test this, we examined germline promoter activity in thymocytes of *Rorc*$^{-/-}$ mice, since in these mice the majority of DP thymocytes would have lost the TEA promoter due to primary V_α to J_α rearrangement, but would retain the central portion of the J_α array in germline configuration. As compared to Rxβ thymocytes, total C_α-containing transcripts were downregulated in *Rorc*$^{-/-}$ thymocytes (**Fig. 7c**), although not to the same extent as in ΔTEAΔJ49Rxβ thymocytes (**Fig. 7b**). Blunted downregulation likely occurs because during primary rearrangement in *Rorc*$^{-/-}$ thymocytes the TEA promoter is replaced by a V_α promoter, rather than simply deleted as in ΔTEAΔJ49 thymocytes. Downregulation of $J_\alpha 58$ transcripts is substantial in *Rorc*$^{-/-}$ thymocytes, owing to deletion of both the TEA and the $J_\alpha 58$ promoters during primary rearrangement. However, despite the general downregulation of total C_α-containing transcripts, $J_\alpha 47$, $J_\alpha 42$, and $J_\alpha 37$ transcripts are much more abundant in *Rorc*$^{-/-}$ as compared to Rxβ thymocytes (**Fig. 7c**). Thus, centrally located promoters are induced whether the TEA promoter is eliminated by gene targeting or by primary rearrangement. As such, central promoter induction should provide a physiologically relevant stimulus for the targeting of secondary rearrangements into the central portion of the J_α array.

DISCUSSION

In this study we show that the TEA promoter and a second promoter associated with $J_\alpha 49$ target primary recombination to discrete sets of 5′ J_α segments, and together direct nearly all normal primary recombination events into the J_α array. Numerous studies have implicated germline promoters as local regulators of accessibility[1–4]. TEA was shown to be required for recombination and histone acetylation across a 15 kb window at the 5′ end of the J_α array, and was assumed to exert direct effects on accessibility that extend across, but are limited to, this defined region of chromatin[7,12]. However, due to the presence of redundant downstream elements, the 3′ extent of TEA's influence on accessibility could not have been known with certainty. In fact, our analysis suggests that accessibility control by TEA may be restricted to a much narrower region than was thought previously. Specifically, we found that TEA-dependent primary rearrangements in *Rorc*$^{-/-}$ mice are limited to $J_\alpha 58$, $J_\alpha 57$ and $J_\alpha 56$. Moreover, since these J_α segments are associated with promoters that are themselves TEA-dependent, accessibility mediated directly by TEA could very well be limited to $J_\alpha 61$ only. Although $J_\alpha 61$ is competent for rearrangement, it was excluded from our analysis since it lacks a functional splice site and could not be surveyed by RT-PCR. We assume that TEA promotes accessibility directly at $J_\alpha 61$, but suggest that it may do so indirectly at $J_\alpha 58$, $J_\alpha 57$ and $J_\alpha 56$

due to its ability to potentiate local promoters at these sites. Thus, the direct influence of TEA on recombinase access could extend over a distance of no greater than 2 kb.

Our study suggests that the J_α49 promoter controls the targeting of primary rearrangements to a window spanning J_α50 to J_α45. However, since J_α47 and J_α45 are associated with promoters whose activity is potentiated by the J_α49 promoter, the J_α49 promoter could conceivably exert a direct influence on accessibility at J_α50, J_α49 and J_α48 only. Thus, the true "windows" for accessibility provided by the TEA and J_α49 promoters may extend no more than 1-2 kb. In terms of range, this would make them more like the D_β1 promoter, which exerts local control over D_β1-J_β1 rearrangement at the *Tcrb* locus[15,16]. Because a 1-2 kb window would typically accomodate one to three J_α segments, our findings have important implications for the way in which J_α segments are sequentially activated during DP thymocyte development. The critical remodeling events that target such windows remain to be defined, but likely include both the covalent modification of histone residues and noncovalent changes in nucleosome structure or organization[11].

Our data emphasize that primary rearrangements are restricted almost exclusively to the region spanning J_α61 to J_α45, and that use of J_α segments downstream of J_α45 depends on the process of secondary rearrangement. Speculations about the mechanisms driving secondary rearrangements have presumed dominant roles for either downstream germline J_α promoters, or V_α promoters introduced by V_α to J_α rearrangement. In the developmental windows model, downstream J_α promoters would be activated according to an intrinsic developmental program in DP thymocytes. The analysis of allelic J_α usage in TEA promoter heterozygous mice argued against this model, since even T cells using relatively 3′ J_α segments displayed uncoordinated allelic J_α usage, with the TEA-deficient allele substantially more advanced than the TEA^+ allele[8]. These results were more consistent with the predictions of a V_α promoter-dependent model, in which rearrangements on the two alleles would progress independently, as a function of the position of the previously introduced V_α promoter. Nevertheless, direct evidence in support of either model has been lacking. Our data provides indirect support for the role of newly juxtaposed V_α promoters. The argument is perhaps clearest for J_α48, which rearranges normally in ΔJ49 mice but barely at all in Δ5′ mice. Jα48 rearrangement on ΔJ49 alleles is therefore almost completely dependent on upstream promoters, even though these promoters lack the ability to target primary J_α48 rearrangements. It is difficult to explain J_α48 rearrangement on ΔJ49 alleles without the involvement of an appropriately positioned V_α promoter as an upstream targeting element. Furthermore, the enhanced usage of J_α47 to J_α38 on ΔJ49 versus ΔTEAΔJ49 alleles is most easily explained by such a mechanism as well.

Our data provide strong support for a distinct and previously unappreciated mechanism that operates to target secondary rearrangements into the central portion of the J_α array. As a consequence of TEA promoter deletion, we observed both the induction of J_α promoters and the retargeting of primary recombination events within the central portion of the J_α array. Moreover, the same promoters were induced when the TEA promoter was deleted by primary rearrangement in *Rorc*$^{-/-}$ thymocytes. Since central promoter activation seems almost certain to account for the central targeting of primary rearrangements in 5′ promoter-deleted mice, it follows directly that these promoters should also stimulate secondary rearrangement upon their induction following primary rearrangement in wild-type thymocytes. Although this promoter-deletion and derepression mechanism relies on embedded J_α promoters as in the developmental windows model, they would be activated by prior V_α to J_α rearrangement as opposed to an intrinsic developmental program. Promoter-deletion and derepression could therefore be compatible with the asynchronous rearrangement observed in mice heterozygous for TEA promoter deficiency[8].

The promoter-deletion and derepression pathway and the V_α promoter-dependent pathway could synergize in important ways to efficiently propagate rearrangement events across the J_α array. If the true window of accessibility provided by germline J_α and newly juxtaposed V_α promoters is maximally a few J_α segments, a V_α promoter-dependent pathway would require many rounds of rearrangement before 3′ J_α segments could be used. It seems reasonable that there must be a substantial interval between successive rearrangement events on a single allele, because at each step TCRs must be expressed at the cell surface to allow testing by positive selection. Hence, there may not be sufficient time for a V_α promoter-dependent mechanism, by itself, to provide accessibility to 3′ J_α segments. In this light, the jumps along the array afforded by downstream promoter induction may synergize with the stepwise progress provided by introduced V_α promoters to allow more efficient use of central and 3′ J_α segments.

Although our studies reveal an intriguing hierarchy among germline promoters, the mechanisms that enforce this hierarchy remain to be established. The dominant TEA and J_α49 promoters clearly have positive influences on the activities of weaker promoters immediately downstream. One possibility is that the dominant promoters provide a degree of downstream chromatin remodeling that enhances transcription factor occupancy at the downstream sites[17,18]. Alternatively, the dominant promoters may be uniquely effective in establishing direct contacts with E_α or in some other way establishing a specific physical organizion of the locus that is then taken advantage of by downstream promoters[19]. The potent suppressive effect of the TEA promoter on centrally located promoters can be explained by either of two mechanisms. One possibility is that suppression reflects promoter competition[20,21]; TEA could compete more effectively for interaction with E_α and in this way exclude the downstream promoters from interactions that are critical for their activation. Alternatively, suppression could reflect transcriptional interference[22–24]; abundant TEA-dependent transcription could interfere with transcription factor loading at centrally located promoters. That 5′ promoters can overcome this suppressive effect emphasizes the complexity of hierarchical promoter interactions across the J_α array. Additional studies will be required to fully understand the mechanistic basis for this hierarchy as well as the qualitative and quantitative aspects of promoter function that are critical for its maintentance.

METHODS

Probes for Southern hybridization. The following DNA fragments were generated for use as radiolabeled probes in Southern hybridization: 3′C_δ, an 867 bp *Hind*III-*Pst*I fragment spanning nucleotodes 11817 to 12683 of Genbank file M64239; J_α60-61, a 446 bp *Hpa*I-*Stu*I fragment spanning nucleotides 20991 to 21436 of M64239; Jα53, a 1011 bp *Bgl*II-*Eco*RI fragment spanning nucleotides 27714 to 28724 of M64239; J_α50, a 682 bp fragment generated by PCR using primers 5′-CCTGCAGTGTGTCAGAGGTTC-3′ and 5′-CCACGGCACTATTTAAGGACC-3′; J_α48-47, a 677 bp *Eco*RV-*Nco*I fragment spanning 36623 to 37298 of M64239; and J_α40-39, a 1018 bp fragment generated by PCR using primers 5′-CTCTCAACCCACATGCTTCC-3′ and 5′-CTTCTCCACCTTGCAGTTTG-3′.

DNase I hypersensitivity. Unfractionated thymoctyes (5×10^7) were permeabilized with lysolecithin and treated with DNase I as described[25,26], with minor modifications. DNase I (Worthington Biomedical) was used at 0, 2.5, 5, 10, 12, 14, and 16 U/ml. *Spe*I digested genomic DNA was fractionated on a 0.7% agarose gel and immobilized onto a nylon membrane. Southern hybridization was performed using the J_α50 probe.

5' RACE analysis. 5′ RACE was performed using a GeneRacer™ Kit (Invitrogen) according to the manufacturer's instructions, making use of a C_α-specific 3′ primer (5′-TGGGAGTCAGGCTCTGTCAGTCTT-3′) and a touchdown PCR strategy. PCR products were purified through agarose gels, were cloned using a TOPO TA Cloning Kit for Sequencing

(Invitrogen) according to the manufacturer's instructions, and were sequenced using a Model 3730 DNA Analyzer (Applied Biosystems).

Luciferase assay. Test fragments were inserted into the pXPG firefly luciferase reporter plasmid (a gift from P. Cockerill, University of Leeds, UK)[27]. Human E_α was amplified by PCR using primers 5′-GTCTCCGAATTCCCTCCAGGTGTTTGG-3′ and 5′-CAAGCGCTGAATTCGGTGAGATCAAGG-3 (which include introduced *Eco*RI sites). Following *Eco*RI digestion, the fragment was cloned into the unique *Eco*RI site downstream of the luciferase gene in pXPG (also referred to as LUC) to generate pXPG-E_α (also referred to as LUC-E_α) A promoter fragment was generated by PCR using primers 5′-CTGATGTCCCGGGTGTCTGATATGTGACCC-3′ (with an introduced *Sma*I site) and 5′-GACAGTCAAGCTTGTTCCTTTCCC-3′ (with an introduced *Hind*III site). The 900 bp *Sma*I-*Hind*III fragment was cloned into the unique *Sma*I and *Hind*III sites upstream of the luciferase gene in either pXPG or pXPG-E_α. Jurkat T cells (4×10^6) were co-transfected with 5 μg of CsCl-purified pXPG derivatives and 10 ng of pRL-TK (renilla luciferase plasmid) (Promega) using Superfect® (Qiagen) as recommended by the manufacturer. Cells were cultured at 37°C with 5% CO_2 for 24h, after which cells were lysed and luciferase activity was measured using a Dual Luciferase Kit (Promega).

Generation of TEA and J_α49 promoter knockout mice. Homology arms were generated by PCR using *pfu* polymerase (Stratagene) and were sequenced to confirm PCR fidelity. The long and short arms of pTEAKO extend from nucleotides 12960 to 18300 and 18973 to 20990 of Genbank file M64239. The long arm was introduced between the *Not*I and *Xma*I sites upstream of the *loxP*-flanked phosphoglycerate kinase (PGK) promoter-neomycin resistance (*neo*^{r}) cassette of plasmid PGKneolox2DTA.2 (http://www.fhcrc.org/labs/soriano/vectors/PGKneolox2DTA.2.html)(gift of Y.W. He, Duke University Durham NC), whereas the short arm was introduced between the *Nhe*I and *Eco*RV sites downstream of the cassette. Homologous recombination deletes 678 bp from the TEA promoter. The long and short arms of pJ49KO extend from nucleotides 28711 to 33461 and 34422 to 36622 of Genbank file M64239. The long arm was introduced between the *Sac*II and *Xma*I sites upstream of PGK-*neo*^{r}, whereas the short arm was introduced between the *Hind*III and *Eco*RV sites downstream of PGK-*neo*^{r}. Homologous recombination deletes 961 bp encompassing the J_α49 promoter and gene segment. Targeting constructs were linearized with *Xho*I and electroporated into the EF1 129SvEv/C57B6 ES cell line (gift of F. Alt, Harvard Medical School, Boston MA). ES clones correctly targeted at the TEA promoter were initially screened by PCR and were subsequently confirmed by Southern blot using the 3′C_δ probe in *Bam*HI-*Kpn*I digests and the J_α61-60 probe in *Stu*I digests. Clones correctly targeted at the J_α promoter were similarly screened by PCR and confirmed by Southern blot using the J_α53 probe in *Xba*I digests and the J_α48-47 probe in *Spe*I digests. *Stu*I and *Spe*I polymorphisms between 129 and B6 insured targeting to the 129 allele. Chimeric mice carrying the TEA deletion were bred to CMV-Cre transgenic mice (B6 background) for germline transmission and to delete *neo*^{r}. *Neo*^{r} was removed from J_α49 targeted ES cells by transient expression of Cre recombinase *in vitro*. Targeted ES cells lacking *neo*^{r} were used directly to produce chimeric mice and were bred for germline transmission, or were retargeted for TEA deletion. The doubly targeted ES cells were screened by PCR and confirmed by Southern hybridization using the 3′C_δ probe in *Kpn*I digests. Doubly targeted ES cells were then treated to delete *neo*^{r} *in vitro* or were used directly to produce chimeric mice that were bred to delete *neo*^{r} *in vivo*. ΔTEAΔJ49 and Δ5′ alleles were generated through both strategies. Gene targeted mice were subsequently bred with *Rorc*$^{-/-}$[28], *Rag2*$^{-/-}$xTCRβ transgene[29] and 129 mice. Breeding schemes insured that littermate controls always segregated wild-type 129 alleles. All mice were used in accordance with protocols approved by the Duke University Animal Care and Use Committee.

Flow cytometric analysis. Suspensions of thymocytes and splenocytes (2×10^6) were incubated for 5 min at 4°C with anti-mouse CD16 to minimize non-specific staining. They were then stained for 20 min at 4° with cocktails of monoclonal antibodies and were diluted with 1 ml of cold RPMI containing 10% fetal bovine serum. Pellets were resuspended in 300 μl of PBS containing 1% formaldehyde. Flow cytometric analysis was performed using a FACStar Plus (BD-PharMingen). Antibodies used were fluorescein isothiocyanate (FITC) conjugated anti-mouse CD4 (GK1.5), phycoerythrin (PE) conjugated anti-mouse CD8α (53-6.7), and Cy-Chrome conjugated anti-mouse TCRβ chain (H57-597). FITC conjugated rat IgG2a, PE conjugated rat IgG2, and Cy-Chrome conjugated hamster IgG2 were used as negative controls. All antibodies were purchased from BD Pharmingen.

RT-PCR analysis of J_α usage. RNA was isolated from total thymocytes and splenocytes using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen) and oligo dT primers. PCR conditions for amplifying $V_\alpha8$ (5′-CAGACAGAAGGCCTGGTCAC-3′) to C_α (5′-TGGCGTTGGTCTCTTTGAAG-3′) products were 94°C for 2 min, 33 to 35 cycles of 92°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a 4 min extension at 72°C. PCR products were fractionated on 1.0% agarose gels, transferred to nylon membranes and hybridized with ^{32}P-labeled J_α specific oligonucleotides. Primers, hybridization and washing conditions are provided in **Supplementary Table 1** online. Quantification was performed using a PhosphorImager. Alternatively, PCR products were gel purified and cloned using a TOPO TA Cloning Kit for Sequencing (Invitrogen). Sequence analysis was performed using C_α primer 5′-CGGCACATTGATTTGGGAGTC-3′.

Chromatin immunoprecipitation. Chromatin immunoprecipitation was performed from mononucleosomal DNA as described[30], using rabbit antisera to diacetylated histone H3 (AcH3) and tetraacetylated histone H4 (AcH4) (Upstate Biotechnology), and control rabbit IgG (Sigma-Aldrich). Bound and input DNA was quantified by real-time PCR using a Roche LightCycler and a FastStart DNA Master Syber Green I Kit (Roche Diagnostics). Primers and annealing temperatures are provided in **Supplementary Table 2** online. The PCR program consisted of denaturation for 4 min at 95°C followed 50 cycles of 20 s at 95°C, 20 s at annealing temperature, and 20 s at 72°C. Acetylation values were expressed as (bound/total for experimental)/(bound/total for mouse *B2m*).

RT-PCR analysis of germline transcription. Thymocyte RNA was converted to cDNA using oligo dT primers and SuperScript (Invitrogen) according to the manufacturer's instructions. PCR conditions were 94°C for 5 min, 22 to 32 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec, followed by a 10 min extension at 72°C. *Actb* primers were 5′-GTCAGAAGGACTCCTATGTG-3′ and 5′-TCGTAGATGGGCACAGTGTG-3′. Amplification of germline C_α transcripts was performed using a 3′ C_α primer 5′-GAGTCGGCTCTGTCAGTCTT-3′ in conjunction with 5′ primers for C_α (5′-AGAACCTGCTGTGTACCAGTTA-3′), $J_\alpha58$ (5′-ACTGGGTCTAAGCTGTCATTTG-3′), $J_\alpha47$ (5′-CTTTGGCTTGGGAACCATTTTG-3′), $J_\alpha42$ (5′-GAGGAAGCAATGCAAAGCTAAC-3′), and $J_\alpha37$ (5′-GACTGGGGACAACTTTACAAGT-3′) **(Fig. 7b)**. Upstream $J_\alpha58$ (5′-TGCAAAGCCCTTCAGTGCAGT-3′), $J_\alpha47$ (5′-GTCACAGGAGTTTGAGGCTGT-3′), $J_\alpha42$ (5′-CCCAAATGACTGTGAATTCTGG-3′), and $J_\alpha37$ (5′-AAAGTGCAGCATTGGGGTGTAA-3′) primers were used for analysis on a recombinase positive background so that transcripts of germline J_α segments could be distinguished from those of rearranged J_α segments **(Fig. 7c)**. Following agarose gel electrophoresis and transfer to nylon membranes, PCR products were detected by hybridization with a ^{32}P-labeled *Actb* PCR fragment generated using the 5′ primer noted above and 5′-

AGGTAGTCTGTCAGGTCCCG-3′ as a 3′ primer, or with ^{32}P-labeled C_α oligonucleotide 5′-CGGCACATTGATTTGGGAGTC-3′.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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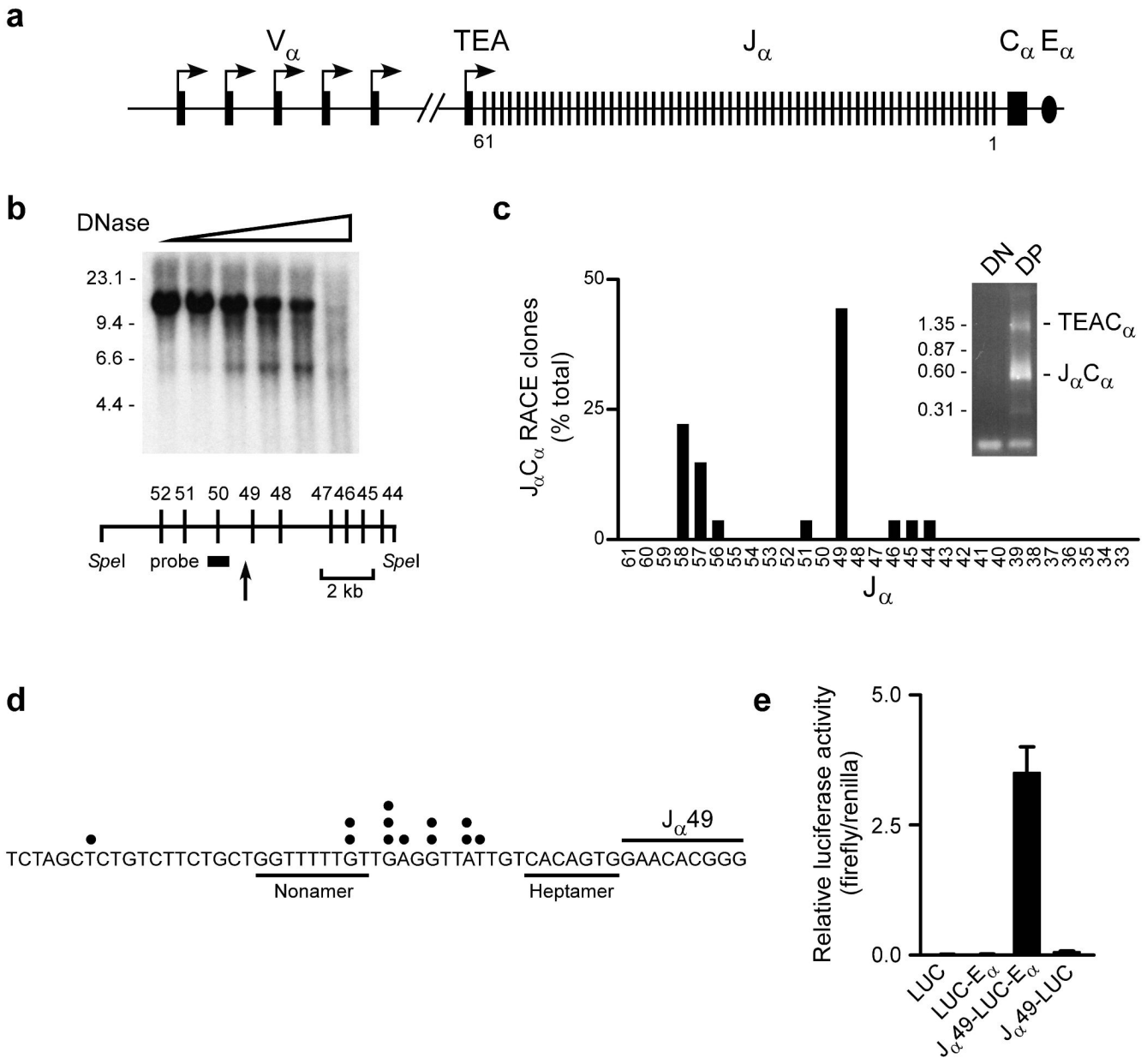


Figure 1.

A promoter upstream of J_α49. (a) Schematic of the *Tcra* locus, identifying V_α(≅100 total), J_α and C_α segments and TEA exon (filled rectangles), V_α and TEA promoters (arrows) and E_α (oval). J_α segment numbering (5′ to 3′) is from 61 to 1. (**b**) Permeabilized Rxβ thymocytes were incubated with increasing amounts of DNase I (wedge) and *Spe*I digested genomic DNA was analyzed by Southern blot. Size markers (in kb) are indicated to the left of the blot. The arrow locates a DNase I hypersensitive site upstream of J_α49. (**c**) RNA of *Rag2*$^{-/-}$ (DN) and Rxβ (DP) thymocytes was subjected to 5′ RACE using a C_α primer. Agarose gel electrophoresis revealed two major species (inset) which were identified by sequencing. Size markers (in kb) are indicated. Frequencies of clones reflecting initiation upstream of or within individual J_α segments is plotted (27 total). (**d**) Map of 5′ ends of RACE clones (dots) initiating near J_α49.

(**e**) A 900 bp promoter fragment was tested for activity in enhancerless (LUC) or enhancer-containing (LUC-E_α) luciferase plasmids. The mean±SEM of five determinations is presented.

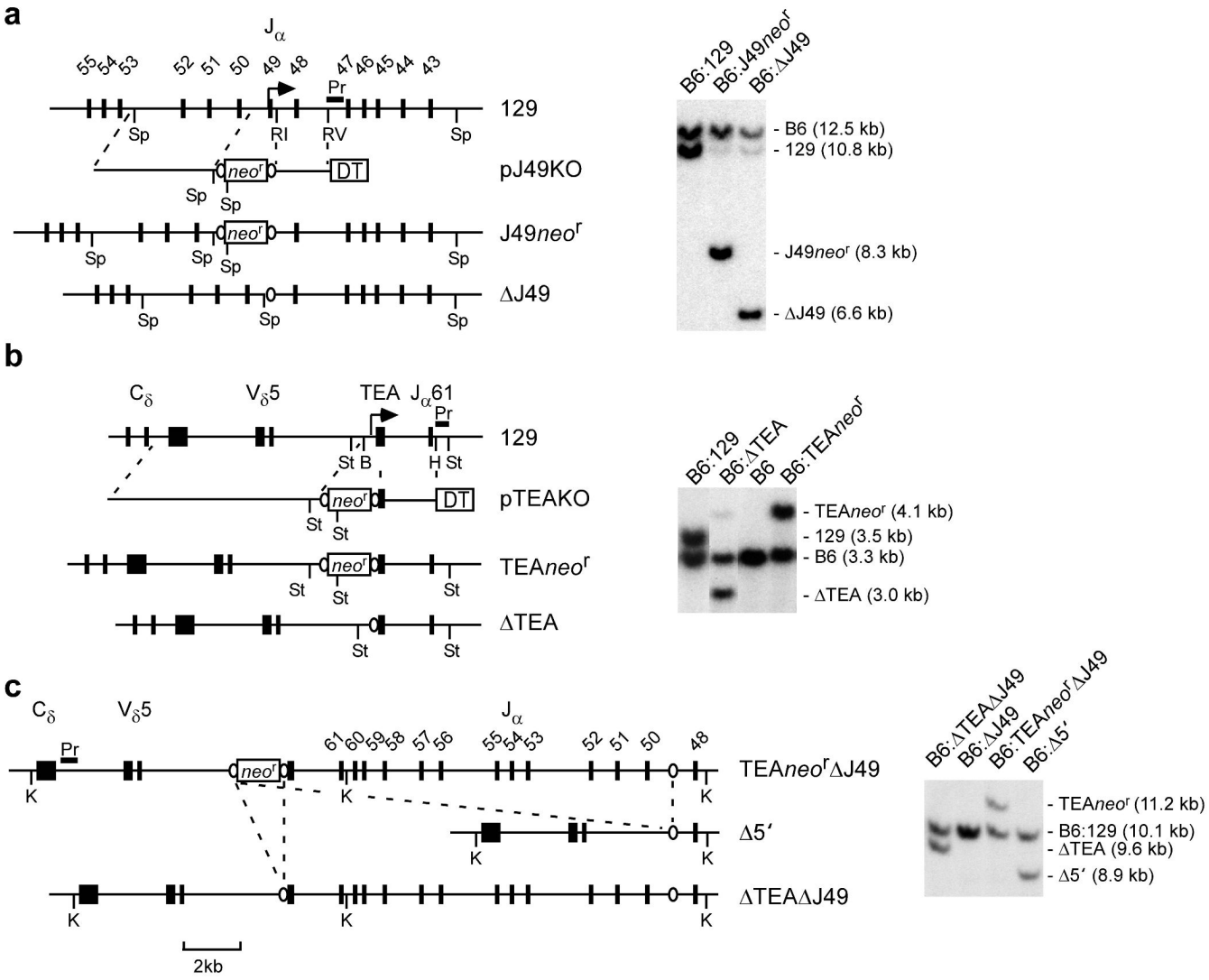


Figure 2.

Generation of TEA and J_{α}49 promoter-deleted mice. Strategies for generation of ΔJ49, ΔTEA, ΔTEAΔJ49 and)5′ mice. V, J, C and TEA exons (filled rectangles) on the wild-type 129 allele are shown, as are neomycin resistance (*neo*^{r}) and diptheria toxin (DT) cassettes (open rectangles) and *loxP* sites (ovals) of targeting constructs pJ49KO and pTEAKO. Restriction sites are: Sp, *Spe*I; RI, *EcoR*I; RV, *EcoR*V; St, *Stu*I; B, *Bgl*II; H, *Hpa*I; K, *Kpn*I. The Southern hybridization probes (Pr) are identified. (**a**) Genomic DNA isolated from B6:129 ES cells, B6:J49*neo*^{r} ES cells, and B6:ΔJ49 ES cells were digested with *Spe*I and analyzed by Southern hybridization. (**b**) B6:TEA*neo*^{r} mice were bred with CMV-Cre transgenic mice (B6 background). As compared to a B6:129 control, Southern hybridization analysis of *Stu*I digested genomic DNA identified progeny carrying a TEA*neo*^{r} allele (B6:TEA*neo*^{r}), two B6 alleles (B6), or a ΔTEA allele (B6:ΔTEA). (**c**) B6:ΔJ49 ES cells were retargeted to introduce a *loxP*-flanked *neo*^{r} cassette in place of TEA (B6:TEA*neo*^{r}ΔJ49). Cre-mediated recombination *in vitro* generated B6:Δ5′ and B6:ΔTEAΔJ49 clones, as revealed by Southern hybridization of *Kpn*I digested genomic DNA.

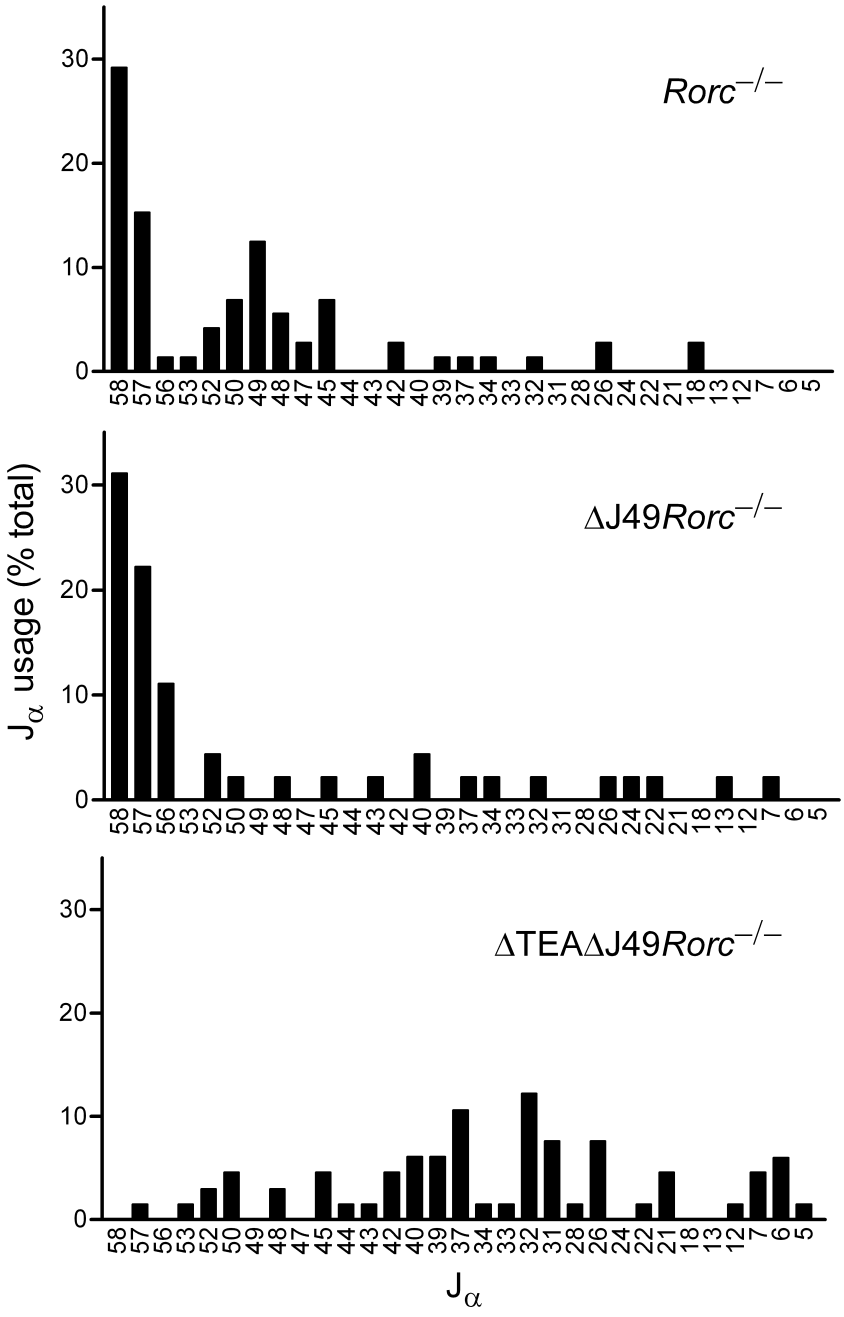


Figure 3.

Primary J_α usage in TEA and J_α49 promoter-deleted mice. V_α8 to C_α RT-PCR products obtained from thymocytes of 4-5 week old *Rorc*$^{-/-}$, ΔJ49*Rorc*$^{-/-}$ and ΔTEAΔJ49*Rorc*$^{-/-}$ mice were cloned and sequenced to evaluate J_α usage. The fraction of clones using particular J_α segments is plotted. Numbers of clones analyzed were 72, 45 and 66 for *Rorc*$^{-/-}$, ΔJ49*Rorc*$^{-/-}$ and ΔTEAΔJ49*Rorc*$^{-/-}$, respectively.

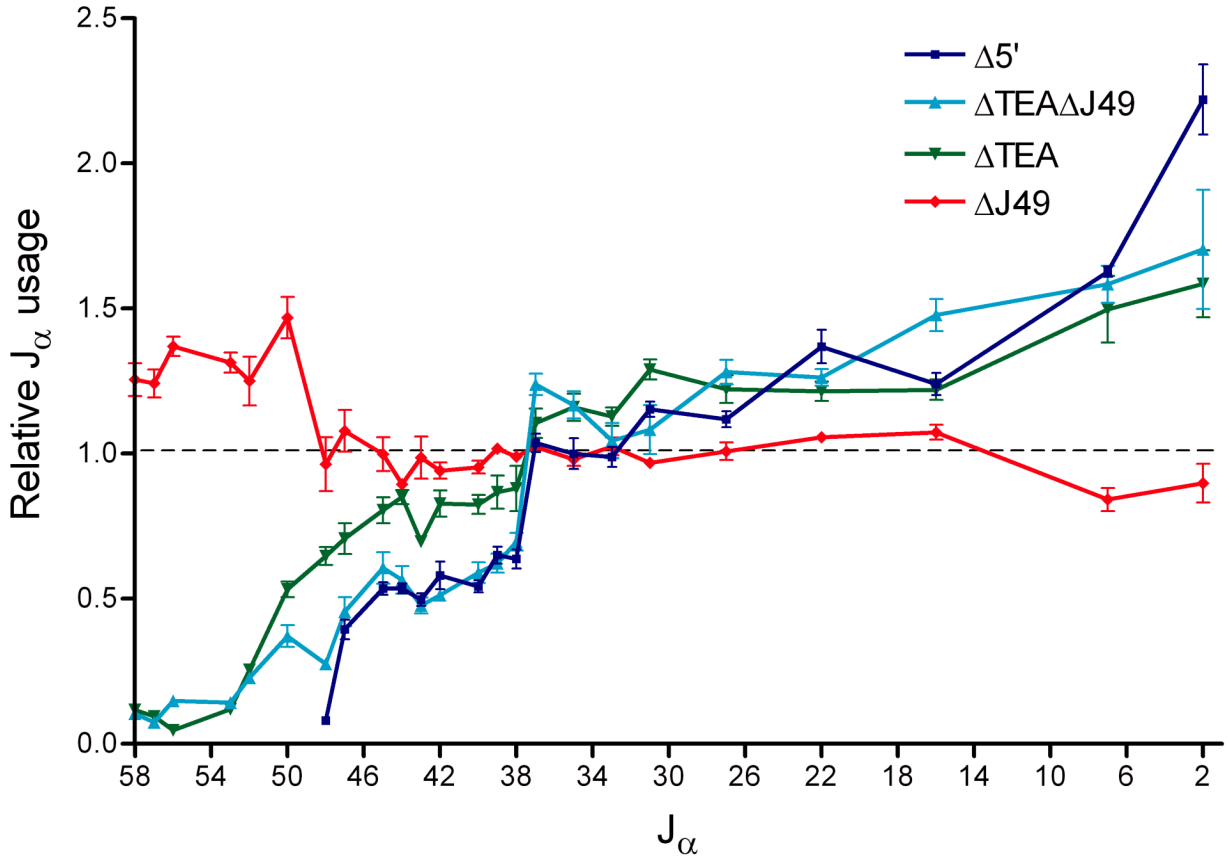
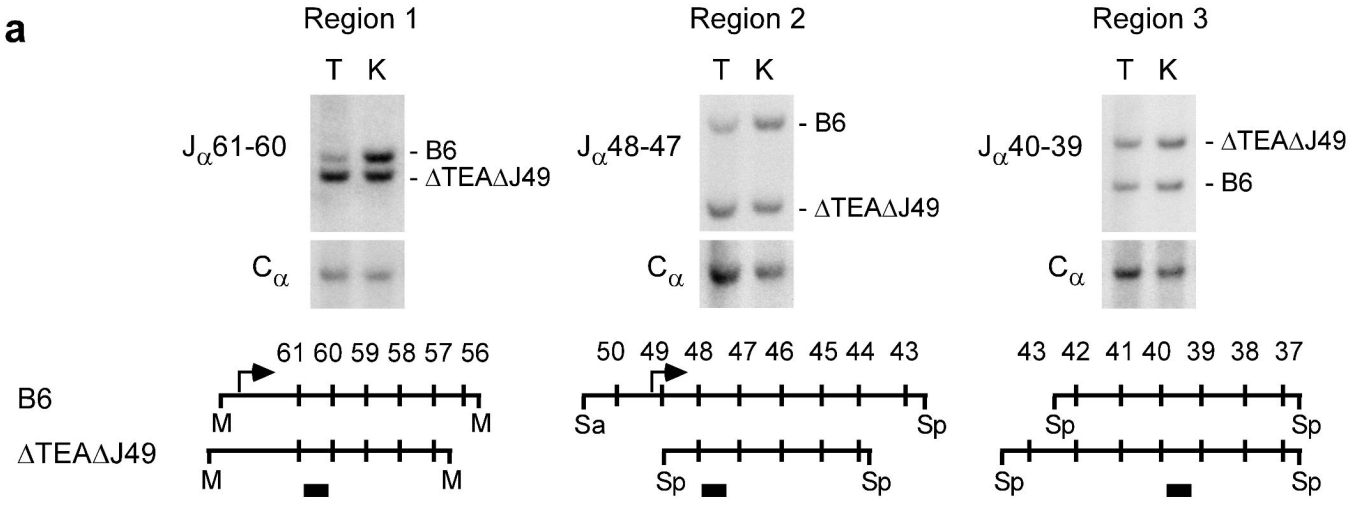


Figure 4.

J_α repertoires of TEA and J_α49 promoter-deleted mice. V_α8 to C_α RT-PCR products obtained from thymocytes of 4-5 week old ΔJ49, ΔTEAΔJ49, and Δ5′ mice, from thymocytes of 2-5 week old ΔTEA mice, and from thymocytes of age-matched wild-type littermates, were fractionated on agarose gels and immobilized to nylon filters. J_α usage was evaluated by hybridization with ^{32}P-labeled J_α and C_α oligonucleotide probes. Relative J_α usage was calculated as (J_α signal for mutant / C_α signal for mutant) / (J_α signal for wild-type / C_α signal for wild-type). The dashed line indicates wild-type J_α usage (frequency=1). Results represent the mean ± SEM of 4-8 determinations.



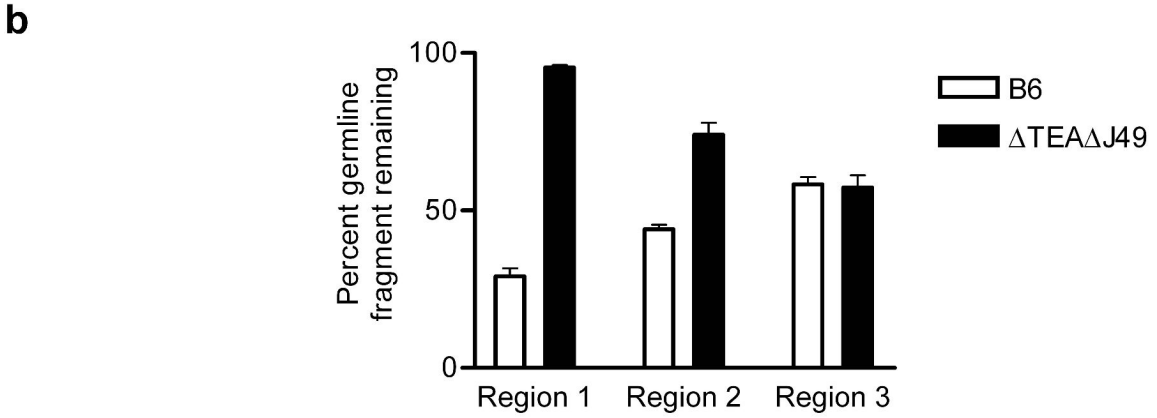


Figure 5.

Quantitative Southern blot analysis of J_α rearrangement on a ΔTEAΔJ49 allele. (**a**) Thymocyte (T) and kidney (K) genomic DNA was isolated from B6:ΔTEAΔJ49 mice and *Msc*I (M), *Spe*I (Sp), or *Sac*I (Sa) plus *Spe*I digests were analyzed by Southern blot using J_α61-60, J_α48-47 and J_α40-39 probes (filled rectangles) and a C_α probe. (**b**) Retention of germline fragment was calculated by first normalizing hybridization signals for J_α to C_α in thymocytes and in kidney. Normalized thymus signals were then expressed as a percentage of the normalized kidney signals. The results represent the mean ± SEM of triplicate determinations.

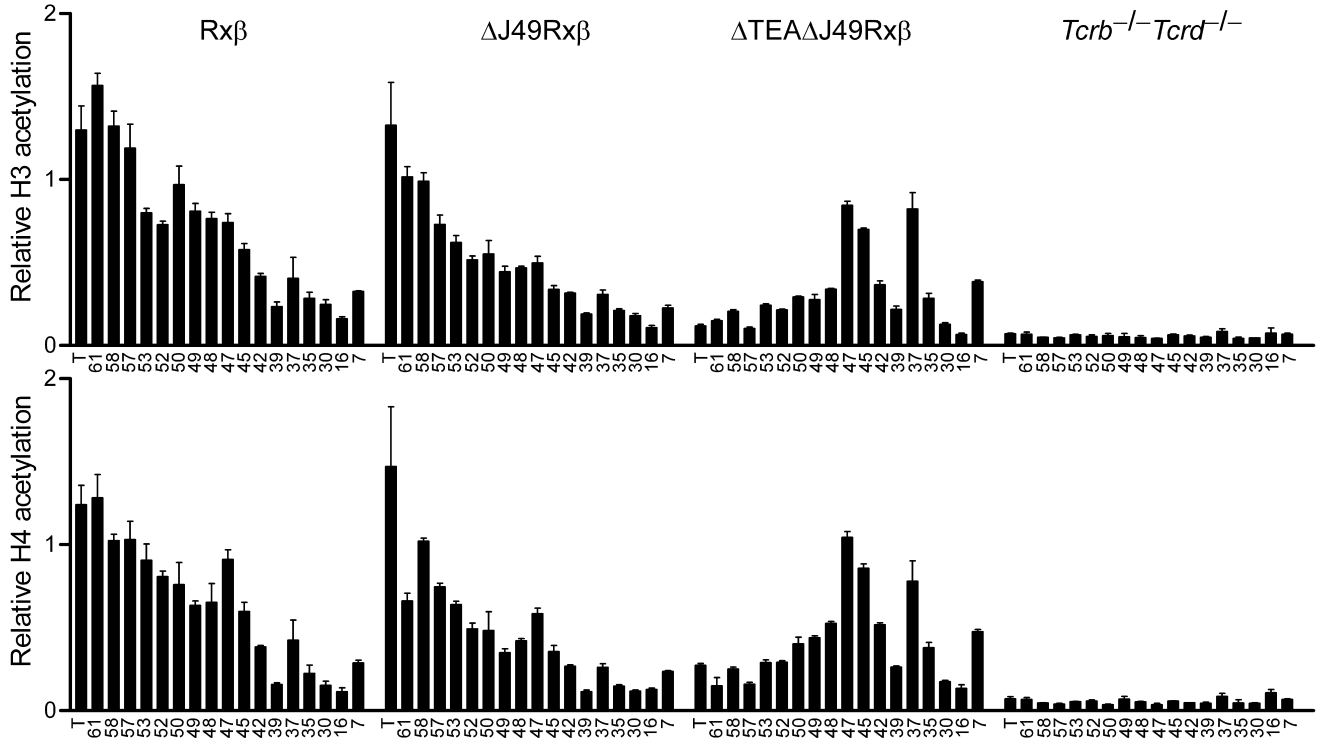


Figure 6.

J_α array histone modifications in TEA and J_α49 promoter-deleted mice. Histone H3 and H4 acetylation was analyzed by chromatin immunoprecipitation from mononucleosomes prepared from thymocytes of Rxβ, ΔJ49Rxβ, and ΔTEAΔJ49Rxβ mice, and as a control, from splenocytes of *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice. Sites analyzed were situated in the TEA exon (T) or at individual J_α segments (numbered). Bound and input fractions were quantified using real time PCR and ratios of bound/input were normalized to that for *B2m* in each sample. The data represent the mean ± SEM of triplicate PCR reactions.

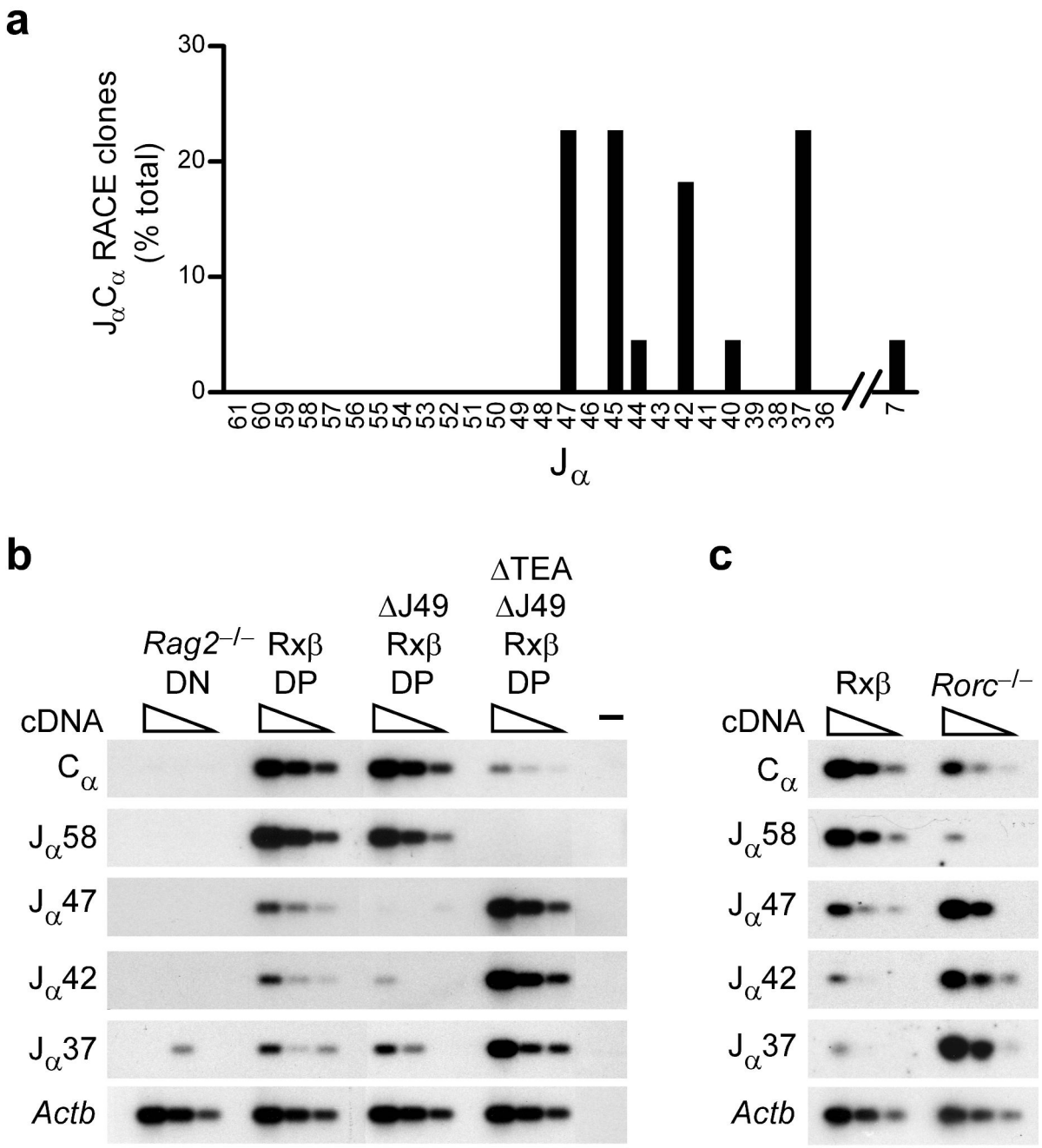


Figure 7.

Germline $J_\alpha C_\alpha$ transcription in TEA and $J_\alpha 49$ promoter-deleted mice. (**a**) RNA isolated from ΔTEAΔJ49Rxβ thymocytes was subjected to 5′ RACE using a C_α primer. The relative frequencies of RACE clones reflecting initiation upstream of or within individual J_α segments is plotted (22 total). Three of five clones identified as $J_\alpha 37$ initiated 66-105 bp downstream of $J_\alpha 37$ and spliced to C_α using a 5′ splice site 129 bp downstream of $J_\alpha 37$. (**b**,**c**) Serial three-fold dilutions of cDNA (wedges) prepared from thymocytes of the indicated genotypes were analyzed by PCR to detect $J_\alpha C_\alpha$ transcripts, total C_α-containing transcripts, or *Actb* transcripts. (-) indicates a control PCR lacking cDNA. PCR reactions were analyzed by Southern blot using C_α or *Actb* probes. The results are representative of two experiments.