Noncoding transcription controls downstream promoters to regulate T-cell receptor α recombination

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The T early α (TEA) promoter in the murine *Tcr* locus generates noncoding transcripts that extend across the 65 kb $J\alpha$ array. Here, we have analyzed the significance of TEA transcription for *Tcr* locus regulation through the targeted introduction of a transcription terminator downstream of the TEA promoter. We demonstrate that noncoding transcription driven by this single promoter can instruct both positively and negatively the activity of downstream $J\alpha$ promoters, and can similarly instruct alterations in $J\alpha$ chromatin structure and $J\alpha$ recombination. TEA transcription activates promoters associated with relatively proximal $J\alpha$ segments and stimulates histone acetylation, histone methylation and chromatin accessibility in this region. In contrast, at more distal locations, TEA transcription inhibits promoter activity through transcriptional interference and suppresses chromatin accessibility. In combination, these effects target initial $V\alpha$ -to- $J\alpha$ recombination to TEA-proximal $J\alpha$ segments and promote the ordered usage of the $J\alpha$ array. The ability of TEA transcription to coordinate the activity of multiple downstream promoters maximizes the biological potential of the $J\alpha$ array and diversifies the *Tcr* repertoire.

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Introduction

Noncoding transcription is thought to serve important functions in gene regulation, and to do so through a variety of mechanisms (Mattick and Makunin, 2006; Prasanth and Spector, 2007). In some settings, the noncoding RNA itself is directly involved in gene silencing or activation (Meister and Tuschl, 2004; Feng *et al.*, 2006; Sanchez-Elsner *et al.*, 2006). In other instances, noncoding transcription, rather than the resultant transcript, is thought to serve a regulatory function. Noncoding transcripts traverse regulatory elements, genes and nonregulatory intergenic regions of a variety of

complex developmentally regulated loci in mammals (Gribnau *et al.*, 2000; Masternak *et al.*, 2003; Bolland *et al.*, 2004; Rogan *et al.*, 2004; Ho *et al.*, 2006). In these instances, noncoding transcription generally correlates with locus activation and is thought to be involved in the establishment of open chromatin domains. This line of thinking is based, in part, on studies demonstrating that elongating RNA polymerase II (RNA pol II) and associated proteins can disrupt chromatin structure and revise the pattern of histone modifications across transcribed coding regions (Belotserkovskaya *et al.*, 2003; Schwabish and Struhl, 2006; Li *et al.*, 2007). Histone modifications introduced by the process of transcription include acetylation, methylation and ubiquitylation. Histone H4 acetylation can disrupt the overall compaction of nucleosome arrays (Shogren-Knaak *et al.*, 2006), whereas H2B ubiquitylation (Pavri *et al.*, 2006) and H3 K36 methylation (Carrozza *et al.*, 2005) are important for the regulated disassembly and redeposition of nucleosomes that occurs during RNA pol II passage. However, because studies of intergenic transcription have often failed to establish causal relationships, it is unclear whether the detected transcription serves a true regulatory function, and what the function might be. In fact, several examples of intergenic transcription that were thought initially to have roles in chromatin opening have more recently been linked to gene silencing via siRNA (Baguet *et al.*, 2005; Haussecker and Proudfoot, 2005).

Noncoding transcription also has the potential to mediate the repression of genes linked in *cis* through a process known as transcriptional interference (Shearwin *et al.*, 2005). In one well-studied example, repression of the *Saccharomyces cerevisiae* *SER3* gene is mediated by regulated transcription across *SER3* from the upstream *SRG1* promoter. *SRG1* transcription can suppress the activity of a downstream promoter by interfering with the binding of transcriptional activators to the promoter (Martens *et al.*, 2004). In other examples of transcriptional interference, transcription from an interfering promoter prevents the transition of RNA pol II from initiation to elongation (Callen *et al.*, 2004) or promotes the release of elongating RNA pol II from the DNA (Prescott and Proudfoot, 2002). While these examples implicate the process of transcription in interference with downstream promoters, a transcribed RNA itself suppresses *dhfr* gene expression by annealing to promoter DNA (Martianov *et al.*, 2007). Recent reports have also suggested that transcriptional interference can repress *Drosophila* *Ubx* (Petruk *et al.*, 2006) and the murine β -like globin gene β h0 (Hu *et al.*, 2007). However, as these studies tested for transcriptional interference by promoter deletion rather than transcriptional blockade, a formal distinction between transcriptional interference and promoter competition could not be made. Well-characterized examples of transcriptional interference in higher eukaryotes are rare.

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V(D)J recombination functions in developing lymphocytes to assemble the variable region exons of both immunoglobulins and T-cell receptors (TCRs) from germline variable (V), diversity (D) and joining (J) gene segments (Cobb *et al*, 2006). The recombinase-activating gene-1 (RAG1) and RAG2 proteins initiate this assembly process by introducing a double-strand break between the coding gene segments and flanking recombination signal sequences (RSSs). Components of the nonhomologous end-joining pathway then join coding ends to form coding joints and signal ends to form signal joints. V(D)J recombination is developmentally regulated, at least in part, via changes in the accessibility of chromosomal RSSs to the recombinase machinery (Cobb *et al*, 2006). Recombinase-accessible chromatin tends to contain hyperacetylated and histone 3 lysine 4 (H3 K4) methylated histones and low levels of DNA methylation. However, a detailed understanding of the chromatin requirements for recombinase access is lacking. The initiation of V(D)J recombination has long been known to coincide with noncoding (or germline) transcription at antigen receptor loci (Yancopoulos and Alt, 1985). However, until recently, the functional relevance of these transcripts had not been established (Abarrategui and Krangel, 2006).

The murine *Tcra* locus undergoes V α -to-J α recombination at the CD4⁺CD8⁺ (double positive, DP) stage of thymocyte development, leading to the production of the α chain of the $\alpha\beta$ TCR. Near the 3' end of the *Tcra* locus is an array of 61 J α segments that occupy a 65-kb region (Krangel *et al*, 2004). Accessibility of these J α segments is controlled by the *Tcra* enhancer (E α) at the extreme 3' end of the locus (Sleckman *et al*, 1997), and two promoters that are activated by E α (Villey *et al*, 1996; Hawwari *et al*, 2005). These promoters, T early α (TEA) at the 5' end of the J α array, and J α 49 15 kb downstream of TEA, produce noncoding transcripts that extend 71 and 56 kb, respectively, across the J α and C α gene segments. E α is required for all V α -to-J α recombination events, whereas the two promoters are required for the targeting of primary V α -to-J α rearrangements to the most 5' J α segments. Subsequently, secondary V α -to-J α rearrangements can replace primary rearrangements through the use of progressively more 5' V α and 3' J α segments (Wang *et al*, 1998; Buch *et al*, 2002). Studies of TEA-deleted alleles have revealed that TEA controls chromatin structure across a 12-kb region at the 5' end of the J α array (Mavieux *et al*, 2003; Hawwari *et al*, 2005; Hawwari and Krangel, 2007). These studies also suggested that TEA is required for the activation of several additional promoters associated with the 5' J α segments and for the suppression of several J α promoters located further 3'. However, it was not clear whether these effects are mediated by TEA transcription or by some other structural or functional property of the TEA promoter region. To initially assess the regulatory function of transcription at the *Tcra* locus, we blocked transcription within the J α array by introducing a transcription terminator downstream of J α 56 (Abarrategui and Krangel, 2006). Transcriptional blockade suppressed recombination of the downstream J α 53 and J α 52 gene segments, and caused an accompanying decrease in H3 K4 methylation at these sites. In addition, transcriptional blockade activated downstream promoters associated with J α 47, J α 42 and J α 37, indicating that these promoters are normally suppressed through a transcriptional interference mechanism.

Here, we introduced a transcription terminator downstream of the TEA promoter to block TEA transcription across the entire length of the J α array. We found that blockade of TEA transcription led to the silencing of additional J α promoters immediately downstream of TEA, but to the activation of J α promoters located further 3'. These perturbations in *Tcra* transcription were accompanied by dramatic changes in chromatin structure and by the retargeting of V α -to-J α recombination events to more 3' J α segments. These results demonstrate that the effects of TEA promoter deletion are due entirely to the loss of TEA transcription and that noncoding transcription driven by a single promoter can have both positive and negative influences on downstream promoter activity and chromatin structure.

Results

Blockade of TEA transcription *in vivo*

To investigate the role of TEA promoter-derived transcription in the regulation of J α chromatin, we used homologous recombination to introduce a transcription terminator cassette into the J α array immediately downstream of the TEA exon (Figure 1). Previous work had established this cassette as a highly effective transcription terminator *in vivo* (Abarrategui and Krangel, 2006). When introduced downstream of J α 56, it caused >99% reduction in steady-state transcript levels as measured by RT-PCR of nuclear RNA and a drop in RNA pol II density to background levels as measured by nuclear run-on.

To analyze the effectiveness of the terminator in the blockade of TEA-derived transcription *in vivo*, mice homozygous for TEA-T alleles ('TEA-T' mice) were bred onto a Rag2^{-/-} $\times$ TCR β transgene (R $\times$ β) background (Shinkai *et al*, 1993). On this background, the absence of Rag2 protein maintains TCR loci in unrearranged configuration, whereas expression of TCR β permits thymocyte development to proceed to the CD4⁺CD8⁺ (DP) stage of development in which the *Tcra* locus is active. Steady-state transcript levels in thymocytes of R $\times$ β and TEA-T R $\times$ β mice were analyzed by RT-PCR of nuclear RNA (Figure 2). As expected, transcript levels upstream of the terminator (TEA) were identical on wild-type and TEA-T alleles. In contrast, transcript levels on TEA-T alleles were reduced to ~4% of wild type immediately downstream of the terminator (5' J α 61), and to ~1% of wild type downstream of J α 56. Transcript levels partially recovered downstream of J α 49, and reached wild-type levels downstream of J α 47, presumably as a consequence of promoter activity associated with these J α segments (Hawwari *et al*, 2005).

TEA transcription regulates J α promoter activity

The low steady-state transcript levels across the 5' J α region suggested that, as we have previously described for Δ TEA alleles (Hawwari *et al*, 2005), the activity of promoters associated with the J α 58, J α 57 and J α 56 gene segments might be compromised on TEA-T alleles. Primary transcripts that initiate at TEA or at J α gene segments extend across the entire J α array to C α , where they are terminated and polyadenylated (Villey *et al*, 1997). Mature transcripts are typically generated by RNA splicing between a donor sequence 3' of either the TEA exon or the first J α segment in the primary transcript, and a splice acceptor 5' of the first C α exon.

Figure 1 *Tcra* locus organization and gene targeting. (A) *Tcra* locus, including the relative positions of V, J and C gene segments and the TEA exon (filled rectangles), promoters (arrows) and Ex (oval). (B) Strategy for the generation of the TEA-T allele. Wild-type 129/Sv allele (129), targeting construct pTEA-T, targeting intermediate allele TEA-T *neo*^f and mutated allele TEA-T. Term, transcriptional terminator; TK, thymidine kinase (open rectangles); ovals, lox P sites. N, *Nco*I sites; Pr, probe. (C) Southern blot of *Nco*I-digested genomic DNA from wild-type (+/+), and targeted (+/TEA-T, TEA-T/TEA-T) mice. Molecular sizes are indicated on the right margin.

Figure 2 Transcription termination in TEA-T mice. In the PCR strategy for analysis of transcription termination (top), arrowheads denote PCR primers. Fivefold serial dilutions (wedges) of cDNA from thymocyte nuclear RNA were PCR amplified and detected by Southern blot and hybridization to radiolabeled oligonucleotide probes to detect unspliced products. Dβ1 transcripts served as loading control. Control PCR reactions: –, no reverse transcriptase; C, water. T (with arrowhead), position of the transcription terminator. Results are representative of two independent experiments.

Figure 3 *Jα* region germline transcription in TEA-T mice. PCR and Southern blot analysis of threefold serial dilutions of thymocyte cDNA to detect spliced germline *Jα* transcripts. TEA-Cα, *Jα*-Cα and total Cα products were detected by Southern blot with a radiolabeled Cα probe. Amplification of *Actb* controlled for cDNA loading. Control PCR reactions: –, no reverse transcriptase; C, water. Results are representative of two independent experiments.

Therefore, as one measure of the activation status of *Jα* promoters in the absence of TEA transcripts, we compared the levels of mature *Jα*-Cα transcripts produced by wild-type and TEA-T alleles by semiquantitative RT-PCR (Figure 3). As expected, mature TEA-Cα transcripts were eliminated on TEA-T alleles due to the transcriptional block imposed by the terminator cassette. Notably, *Jα*58, *Jα*57 and *Jα*56-Cα

transcripts were also undetectable, suggesting that the blockade of TEA transcripts prevents the normal activation of these promoters.

We previously showed that either TEA promoter deletion or the insertion of a transcription terminator downstream of *Jα*56 causes derepression of promoters located in the central portion of the *Jα* array (*Jα*47, *Jα*45, *Jα*42 and *Jα*37) (Hawwari

Figure 4 J α 58, J α 57 and J α 47 promoter activity in TEA-T mice. (A) Agarose gel electrophoresis of 5' RACE products. M, molecular size markers; —, water. (B) Identification of transcription start sites by sequencing of 5' RACE products. Start sites are identified by filled circles and the number of circles over a particular nucleotide indicates the number of times that 5' RACE clone was isolated. Shading identifies the RSS.

et al., 2005; Abarrategui and Krangel, 2006; Hawwari and Krangel, 2007). Consistent with this, J α 49-C α transcripts were increased threefold, and J α 47, J α 42 and J α 37-C α transcripts were increased tenfold on TEA-T alleles as compared to wild-type alleles (Figure 3). These results corroborate our previous data indicating that central J α promoters are normally suppressed through a transcriptional interference mechanism (Abarrategui and Krangel, 2006).

The above analysis is ambiguous with respect to the true initiation sites of the transcripts detected by RT-PCR. As transcripts initiated at TEA span the entire J α array on wild-type alleles, the identified J α 58, J α 57 and J α 56-C α transcripts might reflect alternatively spliced forms of transcripts that initiate at the TEA promoter. Similarly, as transcripts that initiate at the upregulated J α 49 promoter on TEA-T alleles span the central J α segments, the detected J α 47, J α 42 and J α 37-C α transcripts might reflect initiation at J α 49. To eliminate this ambiguity, we mapped J α 58, J α 57 and J α 47 transcription start sites by 5' RACE using total RNA from thymocytes isolated from R $\times$ β and TEA-T R $\times$ β mice (Figure 4A). This approach allows the specific amplification of mature, capped, full-length transcripts within the total mRNA pool. PCR amplification of 5' RACE products of R $\times$ β thymocytes identified a heterogeneous array of initiation sites extending from ~100 to ~500 bp upstream of J α 58

and also identified two main initiation sites upstream of J α 57. These transcripts were not detected in TEA-T R $\times$ β thymocytes. On the contrary, transcripts initiating upstream of J α 47 were detected at low abundance in R $\times$ β thymocytes, but were highly upregulated in TEA-T R $\times$ β thymocytes. Sequencing of cloned 5' RACE products confirmed that the detected transcripts initiated at multiple sites upstream of J α segments, and for all three gene segments identified a fraction of transcripts that initiated within the RSS (Figure 4B). We also independently verified transcription start sites by primer extension of thymocyte RNA from R $\times$ β and TEA-T R $\times$ β mice (Supplementary Figure 1). Taken together, these results demonstrate that transcripts initiating at TEA simultaneously function to activate a set of relatively proximal downstream J α promoters and to repress a set of more distal J α promoters.

Altered J α chromatin structure in TEA-T mice

To understand the effects of transcriptional blockade on J α chromatin structure and function, we used chromatin immunoprecipitation to monitor several histone modifications that are characteristic of active transcription units (Figure 5). On wild-type alleles, diacetylation of histone H3 was highest at the 5' end of the J α array, and gradually decreased toward the 3' J α segments, roughly paralleling the gradient of RNA pol II

Figure 5 *Jα* region histone modifications in TEA-T mice. Analysis of H3 acetylation (A), H3 K4 di- and trimethylation (B, C) and H3 K36 trimethylation (D) by chromatin immunoprecipitation. -3180 and -1700 indicate nucleotide positions upstream of the TEA transcript start site; TEA is positioned 300 nucleotides downstream of the start site. *Oct2*, negative control. *Actb* (exon 4), positive control. Data represent the mean $\pm$ s.e.m. of triplicate PCR. Results are representative of two independent experiments.

density along the *Jα* array (Abarrategui and Krangel, 2006) (Figure 5A). On Δ TEA alleles, H3 acetylation was dramatically reduced from TEA through *Jα*56 and then gradually increased starting at *Jα*53 to reach wild-type levels at *Jα*50. In contrast to Δ TEA alleles, TEA-T alleles revealed wild-type H3 acetylation at TEA. H3 acetylation then diminished gradually across the *Jα* segments just downstream of the terminator, reaching the level observed on Δ TEA alleles by *Jα*57. The disparity in acetylation between TEA-T and Δ TEA alleles at 5' *Jα* segments implies a transcription-independent mechanism for the spreading of this histone modification over several kb from the activated TEA promoter. In support of this idea, H3 diacetylation also diminished gradually upstream of the TEA promoter on TEA-T alleles. On the other hand, the dramatic reductions in acetylation at sites 3' of the terminator on TEA-T as compared to wild-type alleles are solely attributable to blockade of transcriptional elongation. Within the affected portion of the *Jα* array, the decline in H3 acetylation at *Jα*61 should simply reflect the loss of transcriptional readthrough, whereas the declines at *Jα*58, *Jα*57 and *Jα*56 might reflect the combined effects of diminished transcriptional readthrough and the loss of promoter activity at these sites. At central *Jα* segments, the Δ TEA and TEA-T alleles displayed parallel increases in histone acetylation at *Jα*47, *Jα*42 and *Jα*38 as compared to wild-type, consistent with the activation of central *Jα* promoters on both mutant alleles.

Similar analyses of dimethylation and trimethylation at H3 K4 revealed profiles that were almost identical to those for

H3 acetylation (Figure 5B and C). The sole exception was that H3 K4 trimethylation declined sharply both 5' and 3' of the TEA exon on TEA-T alleles. This implies that H3 K4 trimethylation at promoter distal sites can only be acquired through a transcription-dependent mechanism.

Histone H3 K36 methylation is associated with transcriptional elongation (Xiao *et al.*, 2003) and has been shown to recruit the Rpd3S histone deacetylase complex to create a repressive environment that suppresses spurious transcriptional initiation within coding regions (Carrozza *et al.*, 2005). We reasoned that reduced transcriptional readthrough across central *Jα* segments on TEA-T alleles might result in lower H3 K36 trimethylation and a more relaxed chromatin environment that would be permissive for central *Jα* promoter function. On wild-type alleles, H3 K36 trimethylation was moderate at the TEA exon, was substantially elevated at *Jα*61 through *Jα*56 and then decreased significantly at *Jα*53 and across the central *Jα* segments (Figure 5D). As expected, this modification was undetectable on Δ E α alleles. On TEA-T alleles, H3 K36 trimethylation was dramatically reduced from immediately downstream of the terminator through *Jα*50, indicating that its deposition is strictly dependent on transcriptional elongation. Moreover, although H3 K36 trimethylation was detectable at *Jα*47, *Jα*42 and *Jα*38, the level of trimethylation was only about half of that on wild-type alleles. Increased histone acetylation and promoter activity at central *Jα* segments on TEA-T alleles might therefore occur as a consequence of this reduction.

Altered $J\alpha$ accessibility in TEA-T mice

We directly examined the influence of TEA transcription on $J\alpha$ chromatin accessibility by measuring the sensitivity of $J\alpha$ chromatin to DNaseI digestion (Figure 6). Thymocyte nuclei from $R \times \beta$ and TEA-T $R \times \beta$ mice were incubated with increasing amounts of DNaseI, and purified DNA was then

digested with restriction enzymes, blotted and hybridized to radiolabeled control or $J\alpha$ probes (Figure 6A and B). We detected no difference in DNaseI sensitivity of the control *Cd69* gene between the two samples, indicating that digestion conditions were well matched (Figure 6B, bottom). Nevertheless, as compared to wild-type alleles, on TEA-T

Figure 6 $J\alpha$ region accessibility in TEA-T mice. (A) Strategy for Southern blot analysis of $J\alpha$ region DNaseI sensitivity using a 5' $J\alpha$ probe in *SpeI* (S) digests and a central $J\alpha$ probe in *Bam*HI (B) digests. (B) Thymocytes were incubated with DNaseI (5, 10, 15 U) and Southern blots of *SpeI* (left panels) and *Bam*HI (right panels) digested genomic DNA samples were analyzed by hybridization to the indicated $J\alpha$ probes (top panels). Blots were then stripped and reanalyzed by hybridization to a control *Cd69* probe (bottom panels) to insure equal DNaseI digestion and sample loading. $J\alpha$ segments are indicated on the left. –, no DNaseI. Data are representative of two independent experiments (C) In a third experiment, Southern analysis was quantified for loss of the parental *SpeI* (left panels) and *Bam*HI (right panels) fragments using a phosphorimager. Blots were sequentially analyzed with $J\alpha$ and *Cd69* probes.

alleles the parental *SpeI* fragment detected by the 5' J α probe was more resistant to DNaseI digestion, whereas the parental *BamHI* fragment detected by the central J α probe was more sensitive (Figure 6B, top). These conclusions were confirmed by quantitative analysis of an independent experiment (Figure 6C). Thus, transcriptional blockade leads to reduced chromatin accessibility across 5' J α segments and increased accessibility across central J α segments, consistent with the changes in promoter activity and histone modifications at these sites. In addition, blockade of TEA transcription led to the loss of three hypersensitive sites corresponding to the J α 58, J α 57 and J α 56 promoters on TEA-T alleles (Figure 6B, left panels). This suggests a defect in stable transcription factor loading in the absence of readthrough transcription. Nevertheless, at central J α segments, the major hypersensitive sites at J α 49 and J α 47, as well as other minor hypersensitive sites, were detected on both wild-type and TEA-T alleles (Figure 6B, right panels). This suggests that central J α promoters may be at least partially loaded with transcription factors on wild-type alleles even though their transcription is repressed.

TEA transcription controls the targeting of primary rearrangements

We hypothesized that the changes in promoter activity, histone modifications and DNaseI sensitivity at the 5' and central J α segments would translate into differences in RAG protein recognition and cleavage on wild type and TEA-T alleles. To study the role of TEA transcription in the targeting of initial V α -to-J α recombination events in DP thymocytes, we introduced the TEA-T allele onto the *Rorc*^{-/-} background (Sun *et al*, 2000). DP thymocytes from mice deficient for the transcription factor ROR γ are short lived and can support initial, or primary V α -to-J α rearrangement events, but not subsequent, or secondary rearrangement events (Guo *et al*, 2002). We examined J α usage in thymocytes of *Rorc*^{-/-} and TEA-T *Rorc*^{-/-} mice by using V α 8 and C α primers in RT-PCR to amplify the mature transcripts of V α 8-to-J α rearranged alleles. Sequence analysis of the amplified products identified the distribution of J α segments used (Figure 7). Consistent with previous work, primary rearrangements in *Rorc*^{-/-} thymocytes were targeted to J α segments that were immediately downstream of the TEA promoter (J α 58–J α 52), and that surrounded the J α 49 promoter (J α 50–J α 48) (Guo *et al*, 2002; Hawwari *et al*, 2005). However, in thymocytes of TEA-T *Rorc*^{-/-} mice, primary rearrangements to the most 5' J α segments were almost completely abolished (54.5% in *Rorc*^{-/-} versus 0.5% in TEA-T *Rorc*^{-/-} at J α 58–J α 52), whereas primary rearrangements to central J α segments were substantially increased (18.2% in *Rorc*^{-/-} versus 54.8% in TEA-T *Rorc*^{-/-} at J α 47–J α 22). Therefore, transcriptional blockade retargets primary V α -to-J α rearrangements in a fashion that is fully consistent with its effects on J α promoter activity, histone modifications and chromatin accessibility. These results demonstrate that TEA transcription is required to focus initial V α -to-J α rearrangements to the 5' end of the J α array.

Reduced TCR α repertoire in TEA-T mice

We addressed the effect of transcriptional blockade on the steady-state *Tcr α* repertoire using two different approaches. We first analyzed J α usage in mature *Tcr α* transcripts in

Figure 7 Primary J α usage in TEA-T mice. V α 8-C α RT-PCR products of *Rorc*^{-/-} (top) and TEA-T *Rorc*^{-/-} (bottom) mice were cloned and sequenced for analysis of J α usage. Data represent the percentage of clones using the indicated J α segments. Number of clones analyzed: 44 *Rorc*^{-/-} and 42 TEA-T *Rorc*^{-/-}.

thymocytes of wild-type, TEA-T and Δ TEA mice. Following RT-PCR using V α 8 and C α primers, PCR products were blotted and detected by hybridization with radiolabeled J α probes (Figure 8A). Mice with a genomic deletion encompassing TEA to J α 49 (Δ 5') were similarly analyzed to provide a specificity control (Hawwari *et al*, 2005). This analysis revealed a dramatic reduction in usage of J α 58 to J α 52 on TEA-T alleles that was equivalent to the reduction on Δ TEA alleles. Nevertheless, in both instances, J α usage increased to near wild-type levels at J α 49 and more 3' J α segments, a likely consequence of J α 49 and downstream promoter activity. Finally, we more directly examined V α -to-J α rearrangements by PCR of thymocyte genomic DNA using V α 8 and various J α primers (Figure 8B). Consistent with the cDNA analysis, genomic rearrangements of 5' J α segments (J α 61-to-J α 52) were dramatically reduced in TEA-T thymocytes and these reductions were equivalent to those in Δ TEA thymocytes. We conclude that transcription driven by the TEA promoter is critical for the generation of a robust *Tcr α* repertoire.

Discussion

Effects of TEA transcription on J α promoter activity

In the present study, we directly analyzed the function of noncoding TEA transcription by introducing a transcription terminator downstream of the TEA promoter at the 5' end of the J α array. This approach offered a unique opportunity to discriminate the effects of promoter activation from those depending on transcription *per se*. We showed that TEA transcription controls chromatin structure and determines the transcriptional profile of the J α array by promoting the activation of immediately downstream J α promoters and repressing the activity of more distal promoters. It is remarkable that noncoding transcription initiated at a single promoter has opposing effects on different downstream promoters in the J α array. However, the basis for these differential effects is

Figure 8 $J\alpha$ use and genomic rearrangements in TEA-T mice. (A) Southern blot of V α 8-to-C α RT-PCR products prepared from three mice each of the indicated strains and one mouse lacking the 15 kb TEA- $J\alpha$ 49 genomic region (Δ 5'). $J\alpha$ use was determined by hybridization to radiolabeled $J\alpha$ or C α oligonucleotide probes. Analysis of *Actb* controlled for cDNA loading. (–) control PCR lacking cDNA. Data are representative of two independent experiments. (B) Southern blot of twofold serially diluted genomic DNA samples amplified with V α 8 and different $J\alpha$ primers. PCR products were detected using radiolabeled $J\alpha$ oligonucleotide probes. Analysis of *Cd14* controlled for DNA loading. (–) control PCR lacking DNA. Data are representative of two independent experiments.

unclear. One possibility is that the distance between TEA and downstream promoters is the critical parameter that distinguishes activation from suppression, perhaps as a consequence of histone modifications that vary across the length of the transcription unit. Alternatively, differential responsiveness of downstream promoters might rely on intrinsic differences in promoter strength or transcription factor binding.

Positive effects of TEA transcription were restricted to the $J\alpha$ 58, $J\alpha$ 57 and $J\alpha$ 56 promoters. Previous studies of TEA-deleted alleles had suggested that TEA was necessary for transcripts initiating at these $J\alpha$ gene segments (Hawwari *et al*, 2005; Hawwari and Krangel, 2007), and two types of mechanisms had been considered. First, the TEA promoter might act as a physical organizer of the 5' end of the $J\alpha$ array. For example, a physical interaction between TEA and E α might be required to bring the distant E α to the vicinity of the $J\alpha$ 58, $J\alpha$ 57 and $J\alpha$ 56 promoters, so that it could stimulate their activation. Alternatively, transcription initiated at TEA might itself be regulatory. Recapitulation of the Δ TEA phenotype on TEA-T alleles that contain an active TEA promoter offers unambiguous support for a transcription-dependent mechanism.

TEA transcription could influence 5' $J\alpha$ promoter activity if the synthesized RNA itself provided an effector function essential for promoter activation. As an example, a noncoding RNA was shown to mediate recruitment of the transcriptional activator Dlx-2 to the *Dlx-5/6* enhancer in mice (Feng *et al*, 2006). However, this mechanism seems unlikely to stimulate $J\alpha$ 58, $J\alpha$ 57 and $J\alpha$ 56 promoter activity, because there is no obvious sequence conservation near the initiation sites of the three promoters. Alternatively, activation could occur as a consequence of chromatin remodeling events that are linked to transcriptional elongation. Notably, the 5' end of the $J\alpha$ array displays a high density of RNA pol II and elevated levels of several histone modifications that are associated with active and accessible chromatin, including H3 acetylation and H3 K4 di- and trimethylation (Abarrategui and Krangel, 2006). Blockade of TEA transcription led to a dramatic reduction of these histone modifications and resulted in a general decrease in accessibility of 5' $J\alpha$ segments. The observed changes in chromatin structure at $J\alpha$ 61 must reflect direct effects of transcriptional blockade, as $J\alpha$ 61 lacks its own promoter. However, the decreases in chromatin modifications at $J\alpha$ 58, $J\alpha$ 57 and $J\alpha$ 56 segments on TEA-T alleles are likely to reflect both direct effects of blocked transcription and indirect effects of the loss of promoter activity at these sites. Either the specific histone marks introduced by TEA transcription, or the general increase in chromatin accessibility that they provide, could play an important role in activator binding to the $J\alpha$ 58, $J\alpha$ 57 and $J\alpha$ 56 promoters on wild-type alleles. This would be consistent with the reduced DNaseI hypersensitivity at these promoters on TEA-T alleles. Of note, Myc was recently found to bind with high affinity *in vivo* only to binding sites located in regions characterized by high-level H3 acetylation and H3 K4 and K79 methylation (Guccione *et al*, 2006).

Negative effects of TEA transcription were evident in the central region of the $J\alpha$ array. Transcriptional interference is generally believed to depend on the process of transcription without any direct involvement of the synthesized RNA (Shearwin *et al*, 2005). However, it was recently shown that the G/C rich *dhfr* promoter could be repressed *in trans* by an RNA that spanned the promoter (Martianov *et al*, 2007). We do not favor an RNA-mediated mechanism for transcriptional interference at the $J\alpha$ 47, $J\alpha$ 42 and $J\alpha$ 37 promoters, because transcripts initiated at $J\alpha$ 49 also run through the $J\alpha$ 47, $J\alpha$ 42 and $J\alpha$ 37 promoters on TEA-T alleles, but do not repress their activation.

Transcriptional interference by TEA may occur as a consequence of repressive chromatin marks that are introduced

by transcriptional elongation across central J α segments. Although we documented differences in the distribution of H3 K36 trimethylation across the J α array on wild-type and TEA-T alleles, any role for this modification in transcriptional interference at central J α promoters remains uncertain. Alternatively, transcriptional interference by TEA may occur because elongating RNA pol II physically interferes with the initiation of transcription at central J α promoters. The fact that interference occurs with no loss of DNaseI hypersensitivity at J α 47 suggests that it may affect a step that is downstream of initial activator binding. Regardless, any explanation incorporating physical interference by elongating polymerase must address why similar interference is not observed at upstream promoters. We suggest that promoter activation or repression in the J α array might ultimately reflect the balance between two competing effects, transcriptional interference and transcription-dependent chromatin remodeling. Transcriptional interference might have a suppressive influence on downstream promoters along the entire length of the J α array. However, this suppressive effect might be counterbalanced at TEA-proximal J α promoters by potent chromatin remodeling dependent on TEA transcription. The net effect would be conducive to promoter function in the TEA-proximal region, but suppressive to promoter function at greater distances from TEA. We suggest that the opposing influences of transcription documented here are likely to be of general significance, as studies of the human and murine transcriptomes indicate the frequencies of overlapping transcription units to be very high (Carnicini *et al*, 2005; Kimura *et al*, 2006).

Effects of TEA transcription on J α gene segment recombination

Previous studies of TEA promoter-deleted mice showed that TEA is required to promote primary rearrangement of 5' J α gene segments and to suppress primary rearrangement of central J α segments (Hawwari *et al*, 2005; Hawwari and Krangel, 2007). Here, we found that both these effects depend on TEA transcription. Several studies have shown that promoter elements stimulate V(D)J recombination by providing short-range accessibility to nearby RSSs (Sikes *et al*, 2002; Cobb *et al*, 2006; Oestreich *et al*, 2006). In addition, we recently demonstrated that transcription *per se* can provide long-range accessibility and permit the rearrangement of gene segments that are located at some distance from a promoter (Abarrategui and Krangel, 2006). Our results suggest that TEA transcription regulates both types of accessibility at the mouse *Tcra* locus. For example, accessibility at J α 61 depends exclusively on transcription from the TEA promoter situated 1.7 kb upstream. However, accessibility at J α 58, J α 57 and J α 56 is likely to depend both on the long-range accessibility delivered by TEA transcription and by the short-range accessibility provided by local promoters in response to transcriptional readthrough from TEA.

The exact mechanism by which the long-range accessibility is delivered through the process of transcription is not known. Specific histone modifications introduced by transcription are likely to contribute, either by modulating the packing of the nucleosome arrays or by recruiting additional remodeling activities that are in turn important for accessibility (Hassan *et al*, 2001; Belotserkovskaya *et al*, 2003; Pavri *et al*, 2006; Shogren-Knaak *et al*, 2006; Taverna *et al*, 2006;

Wysocka *et al*, 2006). Notably, RAG2 was recently shown to contain a functionally important PHD domain (Elkin *et al*, 2005). Because PHD domains can confer binding to trimethylated H3 K4, this transcription-dependent histone modification could play a direct role in recruiting the recombinase to transcribed RSSs. Alternatively, recombination could be more directly stimulated by transcriptional elongation through any of several mechanisms. A direct interaction between RNA pol II and RAG proteins might deliver the recombinase directly to transcribed RSSs. Moreover, if RAG proteins were to travel with RNA pol II in this fashion, they might then take advantage of the transient nucleosome disassembly that accompanies elongation to access the underlying RSSs (Belotserkovskaya *et al*, 2003; Schwabish and Struhl, 2006). Finally, we cannot rule out the possibility that the synthesized transcript itself might help to recruit RAG proteins to RSSs. Regardless, the work presented here extends our previous data (Abarrategui and Krangel, 2006) and emphasizes that transcription is likely to function broadly as a regulator of accessibility for V(D)J recombination. Understanding precisely how transcription does so will be an important goal of future research.

Materials and methods

Generation of TEA-T mice

Homology arms were PCR amplified using *pfu* Ultra (Stratagene) and PCR fragments were verified by sequencing. The long and short arms extend from nucleotide 12960 to nucleotide 19676 and from nucleotide 19677 to nucleotide 20884, respectively of GeneBank accession number M64239. The transcription terminator, composed of polyadenylation sites followed by a *lac* operator array, has been described elsewhere (Abarrategui and Krangel, 2006). Homology regions were cloned into the pLNTK vector containing *neo^r* and thymidine kinase selection markers driven by the phosphoglycerate kinase promoter. The TC1 129S6/SvEvTAc embryonic stem cell line was transformed by electroporation with the *NotI*-linearized targeting vector. Drug-resistant embryonic stem cell clones were first screened by PCR and correctly targeted clones were then verified by Southern blot using a C δ fragment to probe *KpnI*-digested genomic DNA, and a J α 58 fragment to probe *PvuII*-digested genomic DNA. Chimeric mice were bred with mice expressing a Cre recombinase transgene under the control of the cytomegalovirus promoter to obtain germline transmission and *neo^r* deletion. The TEA-T allele was bred onto *Rag2*^{-/-} $\times$ TCR β transgene (Shinkai *et al*, 1993) (R $\times$ β) and *Rorc*^{-/-} (Sun *et al*, 2000) backgrounds. The wild-type *Tcra* allele carried by R $\times$ β mice was of 129 origin. Mice used for analysis were negative for the Cre recombinase. All mice were used in accordance with protocols approved by the Duke University Animal Care and Use Committee (Durham, NC).

Semiquantitative RT-PCR analysis of germline transcripts

Analysis of unspliced germline transcripts was performed on thymocyte nuclear RNA by semiquantitative RT-PCR as described previously (Abarrategui and Krangel, 2006), with the additional primers 5'TEA-F (5'-AGCTTTGACATCCCTGTCTTCC-3'), 5'TEA-R (5'-TTCTTACAGTGTGCTCCAGGG-3'), 3'TEA-F (5'-ACCTTCCCAGTGAGCAATTTC-3'), 3'TEA-R (5'-ACTCCGTGTTCTCAGAGAGGC-3'). Spliced germline transcripts were analyzed in total RNA by RT-PCR as described previously (Abarrategui and Krangel, 2006).

5' RACE

5' RACE analysis was performed on total thymocyte RNA using the GeneRacerTM Kit (Invitrogen) according to the manufacturer's instructions. Gene specific 3' primers J α 58 (5'-GTTCTGGATGTCTGACTACTGTG-3'), J α 57 (5'-GTTCTGGATGTATGAGCTCACTGTC-3') and J α 47 (5'-GTTCTGGATGTGAGGTCTGACTCTC-3'), which partially overlap the first C α exon, were used for PCR amplification of cDNA products. PCR products were resolved on 2% agarose gels,

were cloned using a TOPO TA Cloning[®] Kit (Invitrogen) and were analyzed by sequencing.

Chromatin immunoprecipitation

Immunoprecipitation of mononucleosomes using anti-diacetylated H3 (Upstate Biotechnology, 06-599), anti-dimethylated H3 K4 (Upstate Biotechnology, 07-030), anti-trimethylated H3 K4 (Abcam, ab8580) and control rabbit-IgG (R&D Systems, ab-105-c) antibodies was performed as described previously (Hawwari *et al.*, 2005). Immunoprecipitations using anti-trimethylated H3 K36 (Abcam, ab9050) were performed as described elsewhere (Bannister *et al.*, 2005). Immunoprecipitated and input material were quantified using a Roche LightCycler and a FastStart DNA Master Syber Green I kit (Roche) by real-time PCR. For immunoprecipitations using anti-diacetylated H3, anti-dimethylated H3 K4 and anti-trimethylated H3K4, analysis of β_2 -microglobulin was used to normalize the ratios of bound/input in different samples. Analysis of *Actb* exon 3 was used to normalize anti-trimethylated H3 K36 immunoprecipitations. Primer sequences are provided in Supplementary Table 1.

DNase I sensitivity assay

Sensitivity of thymocytes to DNaseI digestion was assayed as described elsewhere (Boyes and Felsenfeld, 1996) with a few modifications. Briefly, thymocytes from a whole thymus were isolated and incubated for 5 min on ice with 5 ml of 0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.4, followed by addition of 20 ml of PBS. Thymocytes were then washed with Hanks buffer, resuspended in 0.15 M sucrose, 80 mM KCl, 30 mM HEPES, pH 7.4, 5 mM MgCl₂, 0.5 mM CaCl₂ and 0.067 mg/ml lyssolecithin at 10⁷ cells/ml, and were incubated for 5 min on ice. Thymocytes (3 $\times$ 10⁷) of each genotype were then incubated with DNaseI for 10 min on ice. Digestions were stopped by bringing the reactions to 10 mM EDTA pH 8, 0.4 mg/ml Proteinase K and 0.4% SDS, followed by an overnight incubation at 37°C. Genomic DNA was then

isolated by successive phenol, phenol/chloroform and chloroform extractions, followed by ethanol precipitation. *SpeI* or *BamHI* digests were then fractionated by 0.7% agarose gel electrophoresis, transferred to a nylon membrane and hybridized to either a 5'J α or a central J α probe. To insure equivalent DNaseI digestion of different samples, the blots were stripped and re-hybridized to a *Cd69* probe. The primers used to generate probes were: 5'J α -F (5'-TGGGACCA GAAACCAGGGAG-3'), 5'J α -R (5'-TTTCTGGCCTGGGCTACATCC-3'), central J α -F (5'-AGAGAAGGCATCTTCTAGAGAAGTCC-3'), central J α -R (5'-CTCCAAATCGTGGGGCATTGTTGT-3'), *Cd69*-F (5'-GGTAA AATTGTGAAGTTCCTGTGC-3'), and *Cd69*-R (5'-GTTAGGTGAAGTGGCTTGG-3'). Quantitative analysis of blots was accomplished using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences).

J α usage

Primary J α usage in V α 8 transcripts was analyzed by RT-PCR followed by sequencing as described previously (Hawwari *et al.*, 2005). Steady-state J α usage in V α 8 transcripts was analyzed by RT-PCR followed by hybridization with J α probes as described previously (Hawwari *et al.*, 2005). V α 8-to-J α rearrangements were analyzed using V α 8 and J α primers in genomic PCR as described previously (Abarrategui and Krangel, 2006).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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