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A Barrier-Type Insulator Forms a Boundary between Active and Inactive Chromatin at the Murine TCRβ Locus

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In CD4+CD8− double-negative thymocytes, the murine Tcrb locus is composed of alternating blocks of active and inactive chromatin containing Tcrb gene segments and trypsinogen genes, respectively. Although chromatin structure is appreciated to be critical for regulated recombination and expression of Tcrb gene segments, the molecular mechanisms that maintain the integrity of these differentially regulated Tcrb locus chromatin domains are not understood. We localized a boundary between active and inactive chromatin by mapping chromatin modifications across the interval extending from Prss2 (the most 3′ trypsinogen gene) to Dp1. This boundary, located 6 kb upstream of Dp1, is characterized by a transition from repressive (histone H3 lysine 9 dimethylation [H3K9me2]) to active (histone H3 acetylation [H3ac]) chromatin and is marked by a peak of histone H3 lysine 4 dimethylation (H3K4me2) that colocalizes with a retroviral long terminal repeat (LTR). Histone H3 lysine 4 dimethylation is retained and histone H3 lysine 9 dimethylation fails to spread past the LTR even on alleles lacking the Tcrb enhancer (Ep) suggesting that these features may be determined by the local DNA sequence. Notably, we found that LTR-containing DNA is retained and histone H3 lysine 4 dimethylation fails to spread past the LTR even on alleles lacking the LTR (10). Therefore, we speculate that this transcription, which extends across Dp1, Jp and Cp gene segments, may play an additional role promoting initial opening of the Ep-regulated chromatin domain. The Journal of Immunology, 2011, 186: 3556–3562.

On their cell surface, αβ T lymphocytes express a unique TCR composed of TCRα- and TCRβ-chains that is acquired during T cell development in the thymus. The genetic loci encoding these chains (Tcrα and Tcrb) consist of variable (V), diversity (D), and joining (J) gene segments that are assembled through V(D)J recombination. This recombination reaction is initiated by the lymphoid-specific recombining proteins RAG1 and RAG2, which recognize and generate DNA double-strand breaks at recombination signal sequences that flank V, D, and J gene segments (1). V(D)J recombination occurs in a developmental stage-specific manner, with developmental control exerted in large measure through regulated access of the recombining to selected recombination signal sequences in chromatin (2–4). An accessible chromatin configuration, in turn, is directed by TCR locus promoter and enhancer elements.

The murine Tcrb locus is roughly 700 kb in length and contains 21 functional Vβ gene segments, all but one of which is located upstream of two tandem Dp1-Jp-Cp gene segment clusters (Fig. 1A). The sole exception is the Vβ14 gene segment, which lies downstream of the Cp2 gene segment at the very 3′ end of the locus. A special feature of the Tcrb locus is the arrangement of 19 functional Vβ gene segments in a central cluster that is separated from the 5′ distal Vβ2 gene segments by ~150 kb containing seven trypsinogen genes and gene fragments, and from the Dp1-Jp-Cp clusters by a 250-kb region comprising 13 trypsinogen genes and gene fragments. Notably, whereas Vp1, Dp1, and Jp gene segments are all transcribed and undergo recombination in CD4−CD8− double-negative (DN) thymocytes, trypsinogen genes are not expressed and adopt a relatively closed chromatin conformation in the same cells (5). Hence the locus displays a modular structure with alternating regions of active and inactive chromatin in DN thymocytes.

A Tcrb enhancer (Ep) located between Cp2 and Vp14 works in collaboration with promoters associated with Dp1 and Dp2 to activate the chromatin associated with Dp1 and Jp gene segments (6–10). Deletion of this enhancer severely reduces Dp1-Jp-Cp germline transcription and chromatin modifications, and nearly abolishes Tcrb locus recombination, including the initial Dp1-to-Jp step and the subsequent Vp1-to-Dp1Jp step (6, 7, 9, 10). However, deletion of Ep does not influence transcription and chromatin structure of unarranged Vp1 gene segments, which are activated independently (10). Similarly, Ep does not appear to influence transcription and chromatin structure of the trypsinogen genes, because these genes are inactive in DN thymocytes (5). Rather, the influence of Ep is limited to no more than 25 kb encompassing the two Dp1-Jp-Cp clusters near the 3′ end of the Tcrb locus (10).
In eukaryotic cells, active and inactive genes can coexist and maintain differential regulation despite their proximity to multiple sets of regulatory elements. Insulators prevent the misregulation of neighboring genes by delimiting the chromatin domains over which regulatory elements can function. Two types of insulator elements are involved in the establishment of genomic domains: enhancer-blocking elements that interfere with the ability of enhancers to activate the promoters of neighboring inactive genes, and barrier elements that block the spread of repressive heterochromatin into regions containing active genes (11, 12). In vertebrates, enhancer-blocking is mediated by the DNA-binding protein CTCF (CCCTC-binding factor), which is thought to function by promoting the formation of chromatin loops (13–15). Several mechanisms have been proposed to explain barrier activity, but a common theme is the recruitment of histone-modifying enzymes that deliver activating chromatin modifications that can interrupt the propagation of silent chromatin (16–19). Although transcriptional activity has been demonstrated for many yeast insulators with barrier function, transcription is not an absolute requirement for this function (20, 21).

To ask whether insulator activity helps to maintain the integrity of adjacent regulatory domains of the Tcrb locus, we mapped chromatin modifications to define the border between active Dβ1-IP-Cβ and inactive trypsinogen chromatin. Remarkably, this border, situated ∼6 kb upstream of promoter Dβ1 (P2-D1), corresponded to an endogenous long terminal repeat (LTR). This element was shown to have bona fide barrier activity, because it could insulate an integrated reporter construct from chromosomal position effects in transfected cells. We discuss the implications of our observations in the context of Tcrb locus activation during thymocyte development.

Materials and Methods

Mice

Rag2−/− mice (22), Rag1−/−Eβ−/− mice (10) (kind gift from P. Ferrier, Centre d’Immunologie Marseille-Luminy, Marseille, France), and Rag2−/− × Tcrb transgenic mice (23) were used in accordance with protocols approved by the Duke University Animal Care and Use Committee.

Chromatin immunoprecipitation

Histone modifications were analyzed by chromatin immunoprecipitation (ChIP) using anti-acetylated histone H3 (anti-H3ac; no. 06599; Upstate Biotechnology-Millipore), anti-trimethylated histone H3 lysine 4 (anti-H3K4me3; no. 04745; Upstate Biotechnology-Millipore), and anti-dimethylated H3 lysine 9 (anti-H3K9me2; no. Ab1220; Abcam). ChIP with rabbit IgG (no. AB-105-C; R&D Systems) served as a control. Chromatin was prepared by one of two methods. For some experiments, chromatin was prepared in large scale by micrococcal nuclease digestion and purification of mononucleosomes or a mixture of dinucleosomes and trinucleosomes by sucrose gradient fractionation as described previously (24). For other experiments, chromatin was prepared in small scale by micrococcal nuclease digestion as follows: 1 × 108 thymocytes were washed and lysed as described previously (24). Nuclei were then resuspended in 200 μl digestion buffer, and 5 μl micrococcal nuclease (Worthington) was added for 5 min at 37°C to generate a mixture consisting primarily of mononucleosomes with a small fraction of dinucleosomes. The reaction was stopped by addition of 300 μl of 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 10 mM sodium butyrate, after which the sample was sonicated on ice for eight 15-s intervals using a Sonicator 3000 (Misonix) with the output set to 3.0. After centrifugation, the harvested and Triton X-100 was added to a concentration of 1% (v/v). Chromatin was precleared with protein A-Sepharose/salmon sperm DNA slurry (Upstate Biotechnology-Millipore) and was incubated with the appropriate specific Ab overnight at 4°C followed by addition of protein A-Sepharose/salmon sperm DNA slurry. Bound and input fractions were washed vigorously and DNA was purified. Bound and input fractions were quantified by quantitative real-time PCR using a LightCycler and FastStart DNA Master SYBR Green I kit (Roche) or a Quantifast SYBR Green PCR kit (Qiagen). Ratios of bound to input for modifications H3ac and H3K4me2 were normalized to those for β2-microglobulin; ratios for H3K9me2 were normalized to those for MageA2. PCR reaction conditions were as follows: 5 min at 95°C, followed by 45 cycles of 1 s at 95°C, 5 s at 60–64°C (depending of the primer pair), 7 s at 72°C. PCR primers (Supplemental Table I) matched perfectly only a single Tcrb locus site in the C57BL/6 genome and were judged to amplify unique, single-copy sequences on the basis of melting curve analysis and amplification efficiency from different chromatin.

For analysis of CTCF binding, cross-linked chromatin was prepared and used for ChIP as described previously (25) using 5 μg anti-CTCF Ab (no. 07-729; Upstate Biotechnology-Millipore). Bound and input materials were quantified as described earlier. A known CTCF binding site in the c-myec locus (26) was used as a positive control. PCR primers are shown in Supplemental Table II.

5’ RACE

5’ RACE was performed on thymocyte RNA isolated from a Rag2−/− × Tcrb transgenic mouse using a GeneRacer Kit (Invitrogen) according to the manufacturer’s instructions. Strand-specific primers were used for PCR amplification of cDNA products. PCR products were purified by agarose gel electrophoresis and cloned using a TOPO TA Cloning Kit (Invitrogen). The anti-sense primer tested was 5′-AGCCACAGTGGTGTCGTGCAAGCAG-3′. Two sense primers (5′-CAAAAGCTCAGAGAAAGAGAGTAACAG-3′ and 5′-GATGATGGAGTCAGAGACAG3′) were also tested but did not amplify specific products.

cDNA amplification and analysis

RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer’s specifications. Three micrograms RNA were digested with 3 U RNase-free DNase (Sigma) in 3 μl digestion buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM MgCl2) for 15 min at 23°C. After digestion of EDTA to a final concentration of 2.5 mM and incubation at 68°C for 10 min, the RNA was split in two aliquots that were incubated with SuperScript III (Invitrogen) or mock incubated, according to the manufacturer’s instructions. The resulting cDNAs were diluted 1:10 (or 1:100 for Actb analysis) and used for conventional PCR or for cloning into a pGL3 vector (Promega) containing the mouse minor globin gene (ACACAGATCTT-3′) and P3 (5′-TGGTGACACTTCTGCAG-3′) or quantitative real-time PCR using primers P1 and P2 (5′-ACACAGATCTT-3′ and 5′-GGTCAGAAGGACTCGTCTGCT-3′). Experimental PCR values were normalized to those for Actb (primers 5′-CCTGATGTTAAGAAGACACCG3′ and 5′-CGTCAGAAGGACTCTGATAG3′). To quantify transcripts detected by the P1-P2 primer set relative to total germline Tcrb transcripts, we conducted amplifications with P1-P2 and amplifications with a Cβ primer set (5′-AAAGGCTACCTCTGTGTCG-3′ and 5′-CTGGTGACACTTCTGCAG-3′) from both cDNA and genomic DNA. Percent germline transcripts using the 470-bp exon was estimated as follows: (P1-P2cDNA/P1-P2genomic DNA)/((CβcDNA/Cβ genomic DNA) × 100).

Barrier assay plasmids

To create a reporter construct that would be sensitive to chromosomal position effects, we generated a pGL3 (Promega)-based vector that expressed GFP under the control of a weak promoter (human Vβ1 promoter [PVβ1]) and enhancer (human Tcrd enhancer [Eβ]). The 1.8-kb PVβ1 fragment was excised from plasmid EVSN’s (27) with Sall and HindIII, and cloned into the pGL3-promoter vector digested with XhoI and HindIII. The luciferase gene was then excised by Xbal digestion, followed by Klenow treatment to obtain a blunt end, and subsequent digestion with NheI and GPF gene, isolated from plasmid MS13BP from GPF vector and cloned into the pGL3-promoter vector digested with NcoI, and Klenow treatment, and digestion with NotI, was then introduced into the pGL3 vector in place of luciferase. A 380-bp EcoR1 fragment, obtained from the plasmid EVSN’s by digestion with Xbal and EcoRI, followed by Klenow treatment, was then cloned downstream of GFP by ligation into a BamHI site rendered blunt by Klenow treatment. The resulting plasmid, pGL3-PVβ1-GPF-EcoR1, was used to assay fragments for homologous recombination, with two target sites: one just upstream of the promoter and downstream of the enhancer. Test fragments were amplified by PCR to introduce terminal MluI and NheI sites, and the two PCR fragments were then subcloned stepwise into the multiple cloning site of pGL3-promoter. For cloning into the upstream site, the fragments were excised as a dimer by XhoI digestion, Klenow treatment, and MluI digestion. The excised dimer was then cloned into MluI and Smal digested pGL3-PVβ1-GFP-EcoR1, for cloning into the downstream site, the dimer was excised by digestion with XhoI and MluI, followed by Klenow treatment, and then introduced into the Afel site of the plasmid. Test fragments were: HS4, a 1.2-kb fragment containing the
chicken β-globin 5′ hypersensitive site (HS) 4 insulator (positive control, amplified using 5′-TGAGAACCGCCTCCGGGTACATTACCCGGAGACG-3′ and 5′-AGAGAGCTAGCTCACTTTACGCTCCGTTC-3′); fragment A, a 1.5-kb fragment spanning from 7.6 to 6.1 kb upstream of Db1 (MMAE000665 145657–147165, amplified using primers 5′-ATGCGGCTAGCTCAAGGAGAGACG-3′ and 5′-ATGCCCTAGCTCACTTTACGCTCCGTTC-3′); and fragment B, a 1.5-kb fragment spanning from 6.9 to 5.4 kb upstream of Db1 (MMAE000665 146357–147868, amplified using primers 5′-ATGCACCGCTACTCAAGGAGAGACG-3′ and 5′-ATGCCCTAGCTCACTTTACGCTCCGTTC-3′).

Barrier assay

Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The cells (1 × 10⁶) were transfected with 1 pmol linearized test plasmid plus 0.33 pmol of a linearized puromycin resistance plasmid by electroporation using an Amara Nucleofector system (Lonza), as instructed by the manufacturer. Transfected Jurkat cells were then cultured and expanded in nonselective medium. After 6 d of culture, cells expressing intermediate levels of GFP were sorted on a Beckman-Coulter MoFlo and plated at a density of 1 × 10⁷/ml in selective medium containing 0.2 µg/ml puromycin. After 2 wk of selection, GFP+ cells were resorted using a BD Biosciences FACSVantage (defined as day 0) and cultured in the presence or absence of puromycin for up to 42 d to monitor the loss of GFP expression using a BD Biosciences FACSCanto II.

Results

A 5′ boundary of the Eβ-regulated chromatin domain

Posttranslational modifications of histone tails can reflect the transcriptional status and overall configuration of chromatin: open and transcriptionally active euchromatin is typically marked by acetylation of histone H3 on lysines 9 and 14 and dimethylation of histone H3 on lysine 9, whereas condensed and transcriptionally silent heterochromatin is typically enriched in histone H3 di- and trimethylation at lysine 9 (17, 28–30). To define the edge of the Eβ-regulated chromatin domain, we used ChIP to analyze an 11-kb region spanning from the silent Prss2 gene (the most 3′ trypsinogen gene) to the active PDb1 in DN thymocytes of Rag2−/− mice (Fig. 1A, 1B). We detected a signature of highly active chromatin at PDb1 (H3ac and H3K4me2) and of inactive chromatin at Prss2 (H3K9me2). Notably, there was a sharp transition from H3K9me2 to H3ac over a 1-kb region situated between 5.5 and 6.5 kb upstream of PDb1 and between 2 and 3 kb downstream of the Prss2 polyadenylation signal. Not surprisingly, we detected elevated H3K4me2 that mapped to PDb1 (31). However, we also noted a peak of H3K4me2, centered 6.2 kb upstream of PDb1, that mapped to the transition between H3K9me2 and H3ac. This suggested that chromatin-modifying enzymes may be recruited to this site by an active cis-regulatory element.

Repressive chromatin, as reflected by the H3K9me2 mark, may spread until opposed by chromatin-modifying activities that deliver activating histone modifications such as H3ac and H3K4me2 (32, 33). If this is the case at the Prss2-PDb1 boundary, the activity of Eβ itself could be critical to prevent the encroachment of heterochromatin into the Dβ2-Db1 region. Previous studies showed that, in the absence of Eβ, the Dβ2-Db1 region is characterized by a loss of germline transcription that is associated with reduced histone acetylation, increased DNA methylation, and resistance to nuclease digestion (10). Increased H3K9me2 was also detected at both PDb1 and Cb1 (31).

To determine whether these changes reflected the spreading of heterochromatin from the 5′ boundary, we compared histone modifications across this region in DN thymocytes of Rag2−/− mice in the presence or absence of Eβ. Eβ deletion abolished H3ac in the immediate vicinity of PDb1 and partially reduced H3ac 5.5 and 3.4 kb upstream of PDb1 (Fig. 2A). Eβ deletion also abolished

![FIGURE 1](https://www.jimmunol.org/DownloadedFrom/http://www.jimmunol.org/)

A. A boundary between active and inactive chromatin upstream of PDb1. A, Schematic diagram of the Tcrb locus, including functional Tcrb gene segments (black rectangles) and intervening regions containing trypsinogen genes and gene fragments (gray bars). The Tcrb enhancer (Eβ) (filled oval) and germline PB1 (bent arrow) are also identified. B, A map of H3ac, H3K4me2, and H3K9me2 histone modifications across the Prss2-PDb1 interval in DN thymocytes. H3ac and H3K4me2 were analyzed by ChIP from pure mononucleosomes and H3K9me2 was analyzed by ChIP from pure dinucleosomes and trinucleosomes. Real-time PCR was used to evaluate fold enrichment relative to input, with normalization to β2-microglobulin (B2m) for H3ac and H3K4me2 (right y-axis) and to MageA2 for H3K9me2 (left y-axis). Data are the mean ± SE of two independent experiments. Relative positioning of PDb1, Prss2 (Trypsinogen 20), and previously described DNase HSs (45) are indicated below the graph.

![FIGURE 2](https://www.jimmunol.org/DownloadedFrom/http://www.jimmunol.org/)

A. A boundary maintained in the absence of Eβ. Histone modifications were analyzed on wild-type (WT) alleles in Rag2−/− DN thymocytes and on Eβ-deleted alleles (ΔEβ) in Rag1−/− Eβ−/− DN thymocytes. A, H3ac was analyzed by ChIP from small-scale chromatin preparations. Data are the mean ± SE of three independent experiments. B, H3K4me2 was analyzed by ChIP from mononucleosomes. Data are the mean ± SE of two independent experiments. Data for WT were derived from Fig. 1. C, H3K9me2 was analyzed by ChIP from small-scale chromatin preparations. Data are the mean ± SE of three independent experiments.
H3K4me2 at D\textsubscript{B1}, but this modification was unperturbed at the 5’ boundary (Fig. 2B). Of note, E\textsubscript{B1}-deficient alleles displayed a small increase in H3K9me2 at PD\textsubscript{A1}, but no increase in H3K9me2 in the region between the 5’ boundary and PD\textsubscript{A1} (Fig. 2C). This indicates that E\textsubscript{B1} plays no role in halting the spread of heterochromatin. Rather, if such an activity is present, it is likely mediated by an autonomous element that maintains an active chromatin configuration at the boundary.

An endogenous retroviral LTR at the 5’ boundary

Although a barrier effect may be achieved by disruption of a nucleosome array (34) or attachment to nuclear structures (35), many of the best described barriers including the silent mating-type loci HMR and HML in yeast (16, 36) and chicken β-globin 5’ HS4 (18) share the property that they are highly enriched in histone modifications associated with transcriptional activation (H3ac, H4ac, and H3K4me2). However, transcription itself has been shown not to be necessary for barrier activity (21).

Because the heterochromatin-to-euchromatin transition at the 5’ boundary of the E\textsubscript{B} regulatory domain coincided with a discrete H3K4me2 peak, we asked whether a transcription unit mapped to this region. Accordingly, we searched for transcripts that could initiate in the region by analyzing thymocyte RNA in a 5’-RACE assay using a variety of specific primers. We were unable to amplify PCR fragments using primers designed to detect antisense (relative to the orientation of D\textsubscript{B1}-J\textsubscript{B1}-C\textsubscript{B1}) transcripts (data not shown). However, we did detect and clone several PCR products indicative of sense transcription initiating within a 50-bp region ∼5.7 kb upstream of D\textsubscript{B1} (Fig. 3A).

Analysis of the DNA sequence around the start sites revealed the presence of an endogenous retroviral LTR (LTR BglII family endogenous retrovirus K [ERVK]) (Fig. 3A). This retroviral LTR is 397 bp in length and contains several putative CCAAT and TATA boxes. It is flanked by a 750-bp long interspersed element (LINE) Mur3 L1 fragment that is affixed at its 3’ end to another 365-bp LTR11 family ERVK. The detected transcripts initiate near the junction between the 5’ LTR and the LINE element, whereas the peak of H3K4me2 is centered further upstream at the 5’ edge of the 5’ LTR.

To more fully characterize the structure of these transcripts, we carried out RT-PCR using a forward primer (P1) that anneals across the LTR-LINE junction (to ensure specificity) and a reverse primer (P3) that anneals to C\textsubscript{B1} (Fig. 3B). We identified two major products that were shown by cloning and sequencing to correspond to differentially spliced versions of primary transcripts that run from the LTR at least 11 kb through C\textsubscript{B1} (Fig. 3B, 3C). The transcripts include three novel exons of 470, 780, and 72 bp, which are defined by splice sites that conform well to the 5’ (Δ\textsubscript{B1}-AG-GT\textsuperscript{C}/GAGT) and 3’ (CAG-G) splice-site consensus sequences (Fig. 3B, 3C). The 3’ end of the 780-bp exon aligns with J\textsubscript{B1}2; to the best of our knowledge, the remaining splice sites have not been described previously.

To evaluate the regulation of transcript expression, we analyzed cDNAs prepared from several RNA sources by quantitative real-time PCR (Fig. 3D).

**FIGURE 3.** Repetitive elements and transcription associated with the boundary region. *A.* 5’-RACE analysis. The diagram identifies LTR and LINE elements in the vicinity of the boundary. Numbering represents distance (kb) upstream of D\textsubscript{B1}. The 5’ ends of individual RACE clones are identified by bent arrows in the sequence near the junction of LTR Bgl II ERVK (white lettering on gray background) and LINE Mur3 L1 (black lettering on white background). *B.* Structure of boundary-associated transcripts in DN thymocytes. Transcripts running through C\textsubscript{B1} were amplified by PCR using primers P1 and P3 (arrowheads, left panel). Agarose gel electrophoresis and ethidium bromide staining of the predominant PCR products is shown (right panel). The splicing patterns that give rise to the two products, as deduced from sequencing, are indicated (left panel). *C.* DNA sequence of boundary-associated transcripts. Sequences that define the 470-, 780-, and 72-bp exons are shown. Exon sequences are capitalized and boxed; splicing signals at the intronic borders are in small letters. *D.* Quantification of LTR-derived transcripts. Primers P1 and P2 were used in quantitative real-time RT-PCR. RNA samples were prepared from Rag2\textsuperscript{−/−} thymocytes (DN), Rag1\textsuperscript{−/−} E\textsubscript{B1}−/− thymocytes (ΔE\textsubscript{B1} DN), Rag2\textsuperscript{−/−} × Tcrb transgenic thymocytes (double positive), and Rag2\textsuperscript{−/−} × Tcrb transgenic splenocytes (non-T). Values for the P1-P2 amplicon were normalized to those for Actb and are expressed relative to the value for DN thymocytes (set = 1). Data represent the mean ± SE of two to four independent experiments. *E.* Histone H3K4me3 was analyzed on wild-type (WT) alleles in Rag2\textsuperscript{−/−} DN thymocytes and on E\textsubscript{B1}-deleted alleles (ΔE\textsubscript{B1}) in Rag1\textsuperscript{−/−} E\textsubscript{B1}−/− DN thymocytes by ChIP from small-scale chromatin preparations. Data are the mean ± SE of three independent experiments.
time PCR using primers P1 and P2 (Fig. 3B, 3D). Transcripts were detected in DN thymocytes of Rag2<sup>−/−</sup> mice and in CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes of Rag2<sup>−/−</sup> mice that express a Tcrb transgene (Rag2<sup>−/−</sup>;Tcrb<sup>b</sup>), but were detected at much reduced levels in DN thymocytes of Rag1<sup>−/−</sup> E<sub>b</sub><sup>−/−</sup> mice (ΔE<sub>b</sub> DN) and in splenocytes of Rag2<sup>−/−</sup> Tcrb<sup>b</sup> mice (non-T). Thus, the transcripts are both T cell specific and E<sub>b</sub> dependent. However, even in DN thymocytes of Rag2<sup>−/−</sup> mice, the abundance of these transcripts was low relative to standard germline Tcrb transcripts; by real-time PCR, transcripts detected by P1–P2 were only <0.1% as abundant as those detected by a pair of C<sub>b</sub> primers (data not shown). One caveat is that because primer P1 spans the LTR-LINE junction, it can detect only a fraction of the transcripts initiating in this region (Fig. 3A). However, consistent with low-level transcription, the H3K4me3 modification, whose deposition is tightly linked to transcription (37), was readily detected at PD<sub>9</sub> but was barely detected above background in the LTR-LINE region even on wild-type Tcrb alleles (Fig. 3E). We conclude that H3K4me2 and H3K4me3 deposition are regulated independently in this region, and that neither H3K4me3 nor transcription appears to be involved in forming the identified chromatin boundary.

**Chromatin barrier function of the 5′-LTR**

To determine whether the boundary region contains bona fide barrier activity, we developed a chromosomal position effect assay similar to that used in previous studies (38, 39). This assay relies on a base plasmid in which GFP expression is driven by the human TCRβ enhancer (E<sub>b</sub>) and PV<sub>β</sub>1, with test fragments cloned as dimers both upstream and downstream of the GFP expression cassette (Fig. 4A). Test plasmids were cotransfected into Jurkat cells together with a plasmid conferring puromycin resistance. After selection and sorting for GFP expression, transfected cells were cultured for up to 6 wk in the presence or absence of puromycin. Negative chromosomal position effects were then scored as the loss of GFP expression in a fraction of the transfected cells. Because selection and test plasmids should recombine, GFP loss should not be observed with culture in the presence of puromycin. However, position effects should be revealed during culture in the absence of puromycin, because there should be no selection against silencing at the integration site under these conditions. As expected, in the absence of selection, we found that GFP expression was diminished over time in a fraction of the transfectants when no insulator was present, but was stably maintained when the expression cassette was flanked by the positive control insulator chicken β-globin 5′-HS4 (Fig. 4B, 4C). GFP expression was also lost over time when the expression cassette was flanked by a 1.5-kb test fragment situated upstream of LTR BgIII ERVK (fragment A). However, GFP expression was stably maintained when the cassette was flanked by an overlapping 1.5-kb test fragment that included the LTR (fragment B). Notably, this fragment was as effective as chicken β-globin 5′-HS4. These data support the argument that the LTR functions as a barrier-type insulator that can prevent repressive chromosomal position effects.

**Discussion**

Tcrb locus organization is striking in the sense that it presents alternating blocks of active chromatin composed of Tcrb gene segments and inactive chromatin composed of unrelated Prss genes and gene fragments. This unique organization poses intrinsic regulatory problems, particularly at the borders between active and inactive chromatin. Such is the case at the 3′ end of the locus, where a 250-kb domain of inactive trypsinogen chromatin is juxtaposed to a 25-kb domain of active D<sub>β</sub>3<sub>β</sub>-C<sub>β</sub> chromatin that is regulated by E<sub>b</sub>. In this article, we identified an LTR at the border between these chromatin domains, and showed that LTR-containing DNA functions as a barrier-type insulator that can protect a transgene from negative chromosomal position effects. This LTR colocalizes with a peak of histone modification H3K4me2, suggesting that it may share with other barrier-type insulators the capacity to recruit histone-modifying enzymes that can block the propagation of silent chromatin (16–19). We suggest that by functioning in this way, this LTR helps to maintain the integrity of the E<sub>β</sub>-regulated chromatin domain in the murine Tcrb locus.

Previous studies have revealed that some retroviral LTRs are transcriptionally active, and that they can activate the expression of
neighboring or distant genes (40–42). However, we are unaware of previous instance in which a retroviral LTR was implicated in chromatin barrier activity. We note that although the overall organization of the human Tcrb locus is similar to that of mouse, interspecies sequence conservation in this region is restricted to the Prss2 gene and the PD1 gene only. Nevertheless, the human locus carries a 695-bp LTR (LTR8, family ERV1) that is located 4.6 kb upstream of Dq1. This LTR may serve a similar function in the human locus.

Insulators can encode two separable activities: barrier activity that prevents the spread of repressive chromatin and enhancer-blocking activity that prevents an enhancer on one side of the insulator from activating a promoter on the other (11, 12). The well-studied chicken 5'S-HS4 insulator possesses both activities, with barrier activity mediated by USF1 and enhancer-blocking activity mediated by CTCF. Although our results provide evidence of barrier activity in the Prss2-Dq1 interval, they do not address directly the potential for enhancer-blocking activity. The only vertebrate enhancer-blocking protein identified to date is CTCF (13). We used a CTCF consensus binding-site algorithm (43, 44) to search for potential binding sites in the Prss2-Dq1 interval and found two possible candidates near DNase I HSs 10 and 11 located 2.4 and 3.4 kb upstream of Dq1 (45). However, as compared with positive controls including c-myc (26) and a site between Eβ and Vβ14, ChIP analysis revealed no significant binding of CTCF to these sites or to several additional points tested across the chromatin boundary defined in this study (J. Carabana and M.S. Krangel, unpublished observations). This suggests that the region is unlikely to contain a classical enhancer-blocking element. In contrast, two CTCF binding sites with demonstrated enhancer-blocking activity have been defined just upstream of DFL16.1 in the murine Igh locus (46).

Previous studies of barrier-type insulators HMR and HML in yeast (20, 21) and chicken β-globin 5'S-HS4 (18) have demonstrated that transcription is not required for barrier activity. Nevertheless, we detected low-level, Eβ-dependent and T lineage-specific transcriptional activity associated with the barrier region, with transcripts extending for at least 11 kb across the Dq1-Jγ1-Cβ1 cluster. We do not know whether this transcription, which initiates at the LTR-LINE junction, depends on LTR or LINE sequences. Both elements can have active promoters; moreover, LINE transcripts are unusual because they initiate at the 5' end of the LINE and run through a downstream LINE promoter (47, 48). Further work will be required to clarify this issue. Regardless, we suspect that this transcription is not important for barrier activity, because repressive H3K9me2 chromatin fails to spread in Eβ-deficient mice, even though transcription, which is low on wild-type alleles, is even lower on Eβ-deficient alleles.

We speculate that transcription initiating in the boundary region may serve a distinct and complementary function by promoting opening of the Eβ-regulated chromatin domain. Previous work from Oltz and colleagues (9) demonstrated that maximal chromatin accessibility and histone modifications in the Dq1-Jγ1-Cβ1 region depend on functional interaction between Eβ and PD1, but that Eβ possesses an intrinsic chromatin opening function that is apparent in the absence of PD1. Chromatin opening might then proceed in stepwise fashion, with the initial step dependent on Eβ only, and a subsequent step, resulting in recombinase accessibility, that requires activation of PD1 by Eβ. Because LTR-LINE transcription across the Dq1-Jγ1-Cβ1 region is Eβ dependent, this transcription may provide PD1-independent chromatin remodeling activity that might otherwise be attributed to Eβ only, and that might support developmental activation of PD1. In this regard, recent studies have implicated low-level transcription initiating at a retroviral LTR situated at a distance from the human globin genes in their developmental activation (42). Thus, the boundary region may predispose Dq1-PD1-Cβ1 chromatin to become accessible in two very different ways: it may prevent invasion of repressive histone modifications that would act to oppose chromatin opening, and it may provide a chromatin opening function as well. That said, a chromatin opening function has yet to be demonstrated, and it remains to be proved whether the barrier activity exhibited in our rather artificial Jurkat position effect assay is predictive of similar activity in the context of endogenous cis-regulatory elements at the murine Tcrb locus. Additional experiments will be required to dissect the different functional activities of this element and to assess their biological significance in vivo.

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Disclosures
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References


