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Duality of Enhancer Functioning Mode Revealed in a Reduced TCRβ Gene Enhancer Knockin Mouse Model

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The TCRβ gene enhancer (Eβ) commands TCRβ gene expression through the lifespan of T lymphocytes. Genetic and molecular studies have implied that in early thymocytes, Eβ directs chromatin opening over the Dβ-Jβ-Cβ domains and triggers initial Dβ-Jβ recombination. In mature T cells, Eβ is required for expression of the assembled TCRβ gene. Whether these separate activities rely on distinct Eβ regulatory sequences and involve differing modes of activation is unclear. Using gene targeting in mouse embryonic stem cells, we replaced Eβ by a conserved core fragment (Eβ169). We found that Eβ169-carrying alleles were capable of sustaining β gene expression and the development of mature T cells in homozygous knockin mice. Surprisingly, these procedures and underlying molecular transactions were affected to a wide range of degrees depending on the developmental stage. Early thymocytes barely achieved Dβ-Jβ germline transcription and recombination. In contrast, T cells displayed substantial though heterogeneous levels of VDJ-rearranged TCRβ gene expression. Our results have implications regarding enhancer function in cells of the adaptive immune system and, potentially, TCRβ gene recombination and allelic exclusion. The Journal of Immunology, 2009, 183: 7939–7948.

Transcriptional enhancers act at a distance to orchestrate the spatial and temporal profiles of gene expression in eukaryotes (1, 2). This task is mediated via the binding at discrete sites of a definite collection of specific and general transcription factors (TFs). One key issue is deciphering how an enhancer and bound TFs affect the activity of a cis-linked promoter(s) to integrate signaling cues from within and outside the cell, and, in a constrained chromatin surrounding, convert them into a proper gene response (3). One related and long-standing question is whether an enhancer acts to up-modulate the transcriptional rate at a linked promoter (graded mode) or instead increases the probability of it becoming operational (binary mode) (4). One way to address these issues has been by means of cell transfection or transgenosis using reduced and/or mutated enhancers in various model systems, including Ig and TCR genes (5). Oddly enough, the outcome of such experiments on enhancer function/mode of action has rarely been studied in the native endogenous context.

During B and T lymphocyte development, enhancer/promoter-bound TFs at Ig/TCR loci most likely cooperate to control, via dedicated changes in chromosomal architecture, not only the expression of the given genes, but also their sequential assembly by V(D)J recombination (6, 7). Evidence for such an elaborate partnership has been provided notably by genetic studies at the TCRβ locus (8). In the mouse germline, this locus spreads over ~670 kb, including a ~390-kb 5′ domain containing 21 Vβ gene segments and a 26-kb 3′ domain comprised of a duplicated cluster of Dβ-Jβ-Cβ gene segments, followed by a single Vβ gene segment, Vβ14 (Fig. 1A; also see http://imgt.cines.fr). TCRβ gene recombination is restricted to the T cell lineage and is activated along with locus expression. In CD4+CD8− double-negative (DN) thymocytes (Tcs), this process proceeds in a stepwise manner (Dβ-to-Jβ joining occurring first, before Vβ-to-DJβ assembly), triggering, if productive (in frame), allelic exclusion at the TCRβ locus (9) and further development into the CD4−CD8+ double-positive (DP) cell stage in the αβ T cell lineage, an intricate process also known as β-selection (10).

The TCRβ gene enhancer (Eβ) lies at the center of the ~10-kb Cβ2-Vβ14 intervening region (Fig. 1A). It was first mapped to a 560-bp HpaI-NcoI DNA fragment that conferred T cell-restricted expression to rearranged TCRβ transgenes (11, 12). Gene deletion in a knockout mouse model later revealed Eβ as critical for the efficient onset of cis recombination, with homozygous Eβ-deleted (Eβ−/−) mice displaying impaired TCRβ chain production and αβT cell development (13–15). Further analysis implied that this element, working together with Dβ-upstream promoters of germline transcripts, directs chromatin opening, transcription, and recombination along the adjacent Dβ-Jβ-Cβ clusters.
whereas chromosomal traits and locus expression within the 5' and 3'-located Vβ areas appeared Eβ independent (16–19). Additional evidence implicates Eβ in subsequent expression of VDJ-rearranged TCRβ genes in developed T cells having passed the β-selection checkpoint (20). Knowledge of widespread Eβ activity throughout the T cell lifespan with an apparent requirement in both the early onset and late maintenance of β gene cis expression (including, possibly, fine-tuning in connection to cell selection and/or activation processes) revives the question of whether Eβ function primarily involves a binary or graded mode of gene activation. In the first instance, Eβ would dictate the fraction of TCRβ-expressing cells in a given T lymphoid subset with no impact on actual expression levels. Conversely, in the second case, it would adjust the degree of TCRβ gene expression in a homogeneous manner within the entire cell population. As stressed elsewhere, each mode of action may separately be suited to distinct constraints in terms of developmental choices or adjustable responses, a distinction that might be best assessed via targeted mutation of enhancers in their native context (4).

We tested the consequences of replacing Eβ at the mouse TCRβ locus by a conserved core Eβ fragment (henceforth Eβ(169)) formerly defined using a transgenic approach (21). We report that Tcs from homozygous Eβ(169/169) knockin mice readily develop into mature TCRβ+ T cells. However, TCRβ gene cis expression was affected to a varying degree depending on the stage of T cell development. In DP Tcs and more mature T cells, Eβ(169) alleles generated substantial, although variegated levels of VDJ-rearranged RNA and protein products. In DN Tcs, however, only a few mutated alleles readily achieved V(D)J recombination and germline transcription. Unlike the transgenic approach, Eβ(169) knockin thus unveiled an unanticipated range of enhancer-dependent TCRβ gene responses throughout the T cell life. We relate these behaviors to a latent transition in the Eβ functioning mode, adapted to this enhancer’s operating rationales.

Materials and Methods

Mice

Heterozygous EβWT/169 and homozygous Eβ(169/169) mice were generated, as described previously (14). The targeting vector was similar to the one described by Huang et al. (22), with the exception that the HpaI-NcoI DNA fragment containing Eβ was replaced by a 169-bp NlaIV DNA subfragment (21). C57BL/6J wild-type (WT), Eβ-deleted (Eβ−/−) (14), Rag2-deficient (Rag2−/−) (23), TCRβ-deficient (TCRβ−/−) (24), and SJL mice (25) were also used in this study. Additional mouse models were obtained by strain backcrossing. All mice used in this study were housed under specific-pathogen-free conditions and handled in accordance with French and European directives. All mice were bred on a C57BL/6J background for at least six generations and were killed for analysis between 4 and 6 wk of age.

Flow cytometry and cell purification

Single-cell suspensions of lymphoid cells were prepared and stained with Abs following standard procedures, and were investigated using a FACScan analyzer (BD Biosciences) and FlowJo software (Tree Star). Biotinylated, FITC-, PE-, and allophycocyanin-conjugated mAbs specific for CD4, CD8, CD25, CD45, CD44, CD25, TCRγ, Mac1, Gr1, Ter119, B220, and the IgG1 isotype were purchased from BD Biosciences. Allophycocyanin- and PE-conjugated streptavidin was used to reveal staining with biotinylated mAbs. The Vβ-FITC kit (557004; BD Biosciences) was used to detect specific Vβs. Four-color staining of the investigated cells was achieved, as described previously (15), using either anti-CD4-CD8-TCRβ-Lin (PE mixture of B220, Gr1, Mac1, Ter119) or anti-CD4-CD25-TCRβ-Lin/CD4/CD8(-)-specific Abs. Intracellular TCRβ chain expression (iTCRβ) staining was performed using the Cytofix-Cytoperm reagent (BD Biosciences). Red cells from total spleen suspension were lysed using ammonium chloride, and T cells were isolated using the Pan-T-cell kit (BD Biosciences), followed by autoMACS sorting (Miltenyi Biotech). Three-color staining of the remaining cells was achieved using anti-Thy-β-2I-Lin Abs. Sorting of CD4+ 8 DN and CD4+ 8+ DP Tcs was performed using a FACSVantage cell sorter (BD Biosciences). Lymph node (LN) T cells were isolated using the Pan-T-cell kit and autoMACS sorting. Purity of sorted cells exceeded 98.5%, as assessed by flow cytometry.

RNA and DNA isolation; PCR analyses

RNA/DNA preparations, DNA-PCR, RT-PCR, DNA blotting, and 32P labeling of probes and hybridization procedures were performed, as described previously (16). Each experiment was performed at least twice (including independent T cell sorting) with consistent results.

Hybridoma generation and analysis

Splenic αβ T cells were isolated by autoMACS sorting, stimulated for 72 h using 5 μg/ml Con A, and fused with BW5147 αβ T cells, as described (26). Hypoxanthine/aminopterin/thymidine-resistant T cells were subcloned by limiting dilution and analyzed by FACS for TCRβ and Vβ14 expression. Hybridomas were further investigated by PCR of total genomic DNA for TCRβ allele configuration via the analysis of Dβ1-Jβ1, Dβ1-Jβ2, and Dβ2-Jβ2 rearrangements. Hybridomas were divided into two classes depending on the presence of either one or two VDJ-rearranged alleles, assessed via the presence or lack of germline sequences in the Dβ1 and Vβμ genomic regions.

Analysis of DNA methylation

For bisulfite-assisted analysis of CpG dinucleotide methylation and genomic sequencing, 1 μg of total genomic DNA was converted by bisulfite treatment using the Epitext Bisulfite Kit (Qiagen), as recommended by the manufacturer, and amplified by PCR using oligonucleotide primers designed according to the Urogen methprimer software (http://www.urogene.org/methprimer/index.html). PCR products were gel purified using the Wizard SV Gel Kit (Promega), ligated into pGEM-T easy vector (Promega), and cloned into TOPO10-compotent cells (Invitrogen). Plasmid DNA from selected positive colonies was purified using the Miniprep Wizard Kit (Promega). Following DNA purification, plasmid molecules were sequenced using MWG Biotech sequencing services and T7 primers. Additional quantitative bisulfite sequencing analysis of DNA methylation of the single CpG dinucleotide embedded within the 3' recombination signal sequences (RSS) downstream of Dβ1 (27) used the pyrosequencing technology, as described by DuPont et al. (28), and was performed by Biotage. In each case, 1 μg of purified genomic DNA was used for analysis. Efficiency of bisulfite treatment, assessed via C-T conversion of non-CpG-associated cytosines, was >98%.

Chromatin immunoprecipitation (ChIP)-on-chip

ChIP was performed, as previously described (17), using the anti-H3K4me2 Ab (Upstate Biotechnology). ChIP samples were validated and tested by real-time PCR (quantitative PCR). ChIP-on-chip was performed using the NimbleGen platform (NimbleGen Systems). Briefly, DNA purified from H3K4me2-immunoprecipitated chromatin, along with total genomic DNA, were amplified by ligation-mediated PCR, as described (29). Samples were hybridized to a custom-designed array containing 380,000 probes covering the entire TCRβ locus (plus several additional genomic regions of interest) at a 150-bp resolution. Raw data were visualized using SignalMap software (NimbleGen). Genomic coordinates referred to the National Center for Biotechnology Information mouse genome assembly (build 33).

Oligonucleotides

The oligonucleotide sequences of all the primers and probes used in this study are provided as supplementary information available in Table SII.6.

Results

Definition of the Eβ169 core enhancer fragment

The Eβ 560-bp DNA fragment comprises seven sites (Eβ1 to Eβ7) that bind nuclear proteins in vitro and, for the most part, overlap with motifs specific for lympho/hematopoietic-specialized TFs, including members of the GATA, KLF, bHLH, ETS, and RUNX families (30, 31). Using the DNA methylating agent dimethyl sulfate in vivo, in vivo genomic footprinting assays, we confirmed the occupancy of Eβ3, Eβ4, and Eβ6 in Tcs from WT mice and revealed two new TF-bound sites 5' of Eβ1 and 3' of Eβ7 matching KLF and ETS motifs, respectively (Figs. 1B and S1). In parallel, by

6 The online version of this article contains supplemental material.
phylogenetic footprinting of Eβ sequences from human, mouse, dog, rabbit, and cow, we identified 11 highly conserved motifs, 7 of which corresponded to in vivo footprinted sites, with two additional GATA- and KLF-binding motifs overlapping Eβ1/2, and two bHLH motifs located, respectively, 3′ of Eβ4 and adjacent to the ETS motif on the 3′ end of Eβ (Figs. 1B and S2). Separately, earlier efforts to dissect the functional assets of Eβ have revealed that a reduced enhancer, 169 bp in length (Eβ169), was sufficient to induce recombination within a transgenic TCR minilocus (21). Eβ169 contains nucleotide sequences from 51 bp 5′ of Eβ1 to 22 bp 3′ of Eβ4, and therefore, retains 7 of a total of 11 prospective transcription factor binding sites (TFBS) in Eβ. Moreover, Eβ169 overlaps noncoding sequences that are well conserved throughout the phylogeny (Fig. 1C; 78% homology among all six analyzed species). These data extended our knowledge on Eβ structural content and led us to postulate that Eβ169 might represent a genuine Eβ core enhancer.

**Generation of Eβ169 knockin mice**

To precisely identify any functionally relevant blueprint wired into Eβ169, we used gene-targeting mutational techniques to generate knockin mice in which Eβ169-replaced the Eβ-containing Hpai-NcoI 560-bp DNA fragment (Fig. S3). Homozygous Eβ169/169 mice were born at the expected Mendelian frequency and displayed no apparent differences from WT or heterozygous Eβ1/2 mice, though enabling development of bone fide TCRβ-dependent cell development. Indeed, we also

**FIGURE 1.** Structural organization of the TCRβ locus and enhancer. A. Schematic representation of the mouse TCRβ locus pointing to the relative positions of the Vβ, Dβ, and Jβ gene segments and Cβ exons. Eβ and Dβ1/2 germline promoter cis-regulatory elements are represented by red ovals. Trypsinogen (TG) genes are indicated by red vertical lines. B. Enlargement of the 560-bp Hpai-NcoI DNA fragment containing Eβ and the nuclear protein binding sites Eβ1 to Eβ7 originally identified in vitro. Specific TF-binding motifs within Eβ are indicated on the top; sites found occupied in vivo by genomic footprinting of mouse WT Tcs and the highly conserved motifs (HCM) identified by phylogenetic footprinting are shown on the bottom (see Figs. S1 and S2 for details). C. Pairwise alignments of Eβ orthologous sequences using Mulan tool (http://mulan.dcode.org). The graphs depict the percentages of homology along a 60–100% scale between the mouse and each of the five additional species. The region conserved in all six species is highlighted in red. The Eβ169 location is indicated on the top.

**FIGURE 2.** T cell development in mice homozygous for the Eβ169 mutation. A. WT, Eβ169/169, Eβ169/169;TCRδ−/−, Eβ−/−, and Eβ−/−;TCRδ−/−. LN (top plots) and thymic (middle and bottom plots) cells were analyzed by flow cytometry for surface expression of CD4 vs CD8 and Thy1.2 vs TCRβ, as indicated. Numbers of analyzed cells are indicated on the top, and relative percentages of discrete subpopulations are denoted in the corresponding quadrants. B and C, iτ/TCRβ flow cytometry analysis was performed using the indicated mouse strains and single-cell suspensions from separate thymic cell subsets (B) and peripheral LN CD4+ and CD8+ T cells (C). Inset tables, Report the median fluorescence value (M) and CV of iτ/TCRβ expression for LN cells in the indicated strains of mice. The results shown are representative of three independent experiments.

Altered T cell development in Eβ169 knockin mice

To further evaluate the impact of replacing the Eβ-containing DNA fragment by Eβ169 on T cell development, we performed comparative flow cytometric analyses using total Tcs from WT, Eβ169/169, and Eβ−/− mice. Because TCRδ chain expression allows δ-independent DN-to-TPP cell differentiation (hence masking the developmental defect resulting from the Eβ deletion; see Ref. 15 and references therein), we also used Tcs from Eβ169/169 mice backcrossed onto a TCRδ−/− background. As shown in Fig. 2A (middle plots) and summarized in Table SI, we found CD4+CD8− cells in the Eβ169/169;TCRδ−/− thymus arguing in favor of a Eβ169-driven, TCRβ-dependent cell development. Indeed, we also
noticed that both Eβ^169/169 and Eβ^β/α/β;TCRδ^−/− Tcs developed further into CD4^+ and CD8^+ SP cells in stark contrast to Eβ^−/− or Eβ^β/α;TCRδ^−/− Tcs. However, elevated fractions of DN cells in Eβ^169/169 or Eβ^β/α/β;TCRδ^−/− thymi also implied that Eβ^169 incompletely supports the DN-to- DP cell transition. Consistent with this scenario, Eβ^169/169 and Eβ^β/α/β;TCRδ^−/− DN cells similar to their Eβ^−/− homologues displayed a block at the CD44^+CD25^− (DN3) stage, and Eβ^β/α/β DN cells showed an accumulation of γδT cells (Fig. S5A). Thus, whereas TCRβ-dependent T cell development occurred in mice carrying the Eβ^169 mutation, it did so at a reduced efficiency relative to WT controls. Accordingly, Eβ^169/169 and Eβ^β/α/β;TCRδ^−/− Tcs displayed reduced (but clearly detectable) surface expression of TCRβ chains (Fig. 2A, bottom plots).

**Variegated TCRβ gene expression in Eβ^169 knockin T cells**

Next, we explored iTCRβ in Tc populations from Eβ^β/α/β and Eβ^169/169;TCRδ^−/− mice. Compared with WT controls, we found a quantitative reduction in iTCRβ throughout Tc development in both sets of mice, most compared at the DN3 and DN4 (CD44^+CD25^−) cell stages (Fig. 2B; notice that most Eβ^169/169;TCRδ^−/− Tcs at the latter stage were in fact iTCRβ^−, indicating the presence in this situation, besides a few bona fide β-selected DN4 cells, of a haze of TCRβ^− (presumably not β-selected) CD44^−CD25^− T cells, which was also observed on a Rag^−/− background; also see Fig. S5B and Ref. 32). As expected, iTCRβ was more evident in the mutant Tcs developed to the DP stage and, beyond, in CD4^+ and CD8^+ SP subsets, although in every case it concerned a smaller cell fraction and displayed lower mean intensities compared with WT controls. Although iTCRβ variations between the WT and Eβ^169/169 situations appeared slightly less marked in peripheral T cells, median (M) expression was reduced in the Eβ^169/169 cells (Fig. 2, C and inset tables). These profiles suggest that, in mature T cells, the Eβ^169/169 mutation generally leads to a decrease in TCRβ gene expression. As attested by relative measurements of the coefficient of variation (CV), this defect in the mutant cells coexisted with a wider range of iTCRβ intensities (i.e., increased heterogeneity or variegation).

We extended these observations to T cell hybridomas derived from Eβ^β/α/β splenocytes (e.g., Fig. S6A), thus arguing that the two TCRβ aberrations in the mutant situation (reduced expression and enlarged heterogeneity) occur at the clonal cell level. Taking advantage of sorted TCRβ low or high expressers from these hybridomas, we additionally tested whether TCRβ expression profiles were similarly inherited during cell proliferation in the mutant vs WT context. Over a 5-wk culture period, we noticed that Eβ^β/α/β T cell outliers (either TCRβ^low or TCRβ^high) generally readjusted TCRβ expression levels to the parental profile less efficiently compared with their WT counterparts (Fig. S6B and data not shown). The slow restoration of the parental cell-to-cell variability profile by spontaneous outlier descendants independent of their initial expression levels has recently been proposed to be mainly the result of processes involving stochastic transitions between multiple stable states, thus leading to the observed heterogeneity of protein expression within a clonal population (33). This being the case, our data supporting a longer phase of adjustment to equilibrium by the Eβ^169/169 T cells imply that Eβ replacement by a shorter Eβ^169 fragment appreciably slowed down these stochastic transitions and, potentially, intensified the TCRβ variegated phenotype.

**Biased Vβ repertoire in Eβ^169 knockin mice**

When applied to primary T cells and TCRβ analysis, flow cytomtery portrays a collection of functional VβDJβ-Cβ units, each produced from a single Vβ gene and associated promoter. Therefore, to complete this study, we applied this methodology to a comparative analysis of the Vβ repertoire expressed by Eβ^β/α/β vs WT LN T cells using a panel of anti-Vβ Abs. As shown in Fig. 3A, peripheral T cells from WT mice expressed diverse Vβ chains in variable proportions, in agreement with previous studies (34, 35). Surprisingly, on Eβ^β/α/β T cells, we predominantly detected a small number of distinct Vβ chains in proportions that varied from the WT, suggesting the expression of a biased repertoire in the mutant situation. Distinct Vβs included Vβ_8.1/9.2, Vβ_14, and Vβ_5 (in higher proportions with respect to their homologues in WT T cells) and Vβ_169/169, and Vβ_3 (in near similar proportions), overall
accounting for ~53% of the peripheral T cell pool in the mutant mice (vs 38% in the WT). Several Vβs that were found on almost identical fractions of WT T cells (e.g., Vβ3, Vβ6, Vβ14) displayed dissimilar ratios in the mutant state, arguing against the Eβ169/169 profile simply reflecting a reduced expression of an otherwise physiological Vβ chain distribution. Besides, the biased set of Vβs expressed by the Eβ169/169 T cells is apparently distinct from that reported in instances of unusual T cell subsets such as the NKT cells (36). Importantly, the Eβ169/169 T cell surface expression of the given Vβ isotypes commonly displayed altered M and (to a lesser extent) CV values reminiscent of the profiles found in ic TCRβ-staining experiments (Fig. 3B and data not shown). Overall, these findings imply that the biased Vβ isotopic repertoire expressed by the Eβ169/169 T cells only accounts for part of the observed β-chain heterogeneity. In contrast, the maintenance of altered M and CV values at the single Vβ level validates the view that the reduced enhancer sustained the expression of an assembled β gene at generally lower and varying degrees, consistent with a contribution of a graded, noise-attenuating component in the mode of action of the Eβ element in late developing and mature T cells (see Discussion). As expected, the assessment of RNA expression from a few rearranged Vβ genes in purified LN cells from RT-PCR assays was coherent with the findings of Vβ isotopic heterogeneity. Thus, we detected Vβ8.1, Vβ3.1, and Vβ14 rearranged transcripts in Eβ169/169 T cells, and much less or no Vβ6, Vβ8, or Vβ11 rearranged transcripts (Fig. 3C). Globally, amounts of β transcripts (assessed via the analysis of Cβ expression) were reduced by 2- to 5-fold in Eβ169/169 T cells compared with WT, in general agreement with the icTCRβ (FACS) data.

Altered TCRβ expression and recombination in Eβ169/169 DN thymocytes

The moderate decrease in TCRβ RNA and protein expression found in mature T cells of an Eβ169/169 mouse background contrasted with the drastic block in DN-to-DP T cell transition also observed in these mice. Altered production of TCRβ+ DP Tcs of the Eβ169/169 genotype may be the result of a block in TCRβ gene early expression and/or rearrangement at most Eβ169 alleles before β-selection. To address these issues, we purified DN and DP T cells from WT, Eβ169/169, and Eββ−/− mice and measured in parallel β gene expression and rearrangements using dedicated RT-PCR and DNA PCR assays. Attempts to detect several Vβ rearranged transcripts in Eβ169/169 DN Tcs initially failed (and Cβ and DJFβ-Cβ transcripts were barely seen), whereas all these products were readily amplified in DP Tcs from the same mice using identical assay conditions (Fig. 3, D and E; note the variations in intensity in favor of the Vβ3, Vβ6, and Vβ14-containing transcripts compared with the Vβ6, Vβ8, and Vβ11-associated ones, similar to RT-PCR data from LN T cells). Eventually, in increasing the number of PCR cycles, TCRβ transcripts were detected in Eβ169/169 (but not Eββ−/−) DN Tcs, in a range estimated between ~0.2 and 0.03% of those in WT cells (Fig. 3F). Thus, the proportion of Eβ169/169 early developing T cells expressing their (possibly rearranged) TCRβ genes must have been very low.

In parallel, semiquantitative DNA PCR assays uncovered DJβ1- and Vβ8/5/14(DJ)β-containing coding joint (CJ) products of V(DJ) recombination in Eβ169/169 DN Tcs in amounts that were small, yet noticable when compared with Eββ−/− DN Tcs (Fig. 4A; DJβ2 and VβDJβ2 rearrangement profiles are shown; parallel analysis of DJ1β1/2 and VβDJβ1 CJJs gave consistent results). Interestingly, we found hardly any Vβ11 CJJs in Eβ169/169 DN Tcs, suggesting that the biased Vβ repertoire observed in mutant T cells may, at least in part, be established from the V-to-DJ recombination step onward. Based on dilutions of the WT samples, we estimated the amounts of DJβ1 and Vβ8/5/14(DJ)β rearrangements present in Eβ169/169 DN Tcs to be ~1% of those in WT controls; potentially, a somewhat lesser drop compared with that denoted above for the corresponding transcripts. As expected, we detected the same CJ products more readily in Eβ169/169 DP Tcs, with all except Vβ11 reaching WT levels (Fig. 4B). Together, these results imply that the developmental block (and, possibly, altered β repertoire) of Eβ169/169 DN Tcs was due in part to a low efficiency of V(DJ) recombination at the mutated TCRβ locus. This assertion is supported by the results from DNA PCR analysis of Eβ169/169 T cell hybridomas. As summarized in Table I, among 82 TCRβ+ hybridomas (i.e., exhibiting at least one productively rearranged (VDJ+β) allele), only 9 (11%) carried two fully assembled VDJβ/VβDJβ alleles, considerably less than the ~40% typically observed in a WT context (26, 37). Furthermore, amid the 73 remaining, VβDJβ singly assembled clones, only 11 (~13%) bore the opposite β allele in a DJβ rearranged form, whereas 62 (75%) had it still in the germline Vβ-DJβ-β configuration, contrasting again with the normal situation in which the latter picture is rare (<10%) and the VβDJβ/VβDJβ pattern instead predominates (~60%). In short, DNA rearrangement studies pointed out that, in stark contrast to the normal state, the bulk of Eβ169/169 Tcs that achieved VβDJβ assembly did so following restricted processing of one TCRβ allele and, mostly, one DJβ-β cluster only imposing infrequent V(DJ) recombination events at Eβ169-associated TCRβ loci.

Eβ169 poorly competes with WT Eβ in sustaining cis recombination and β-selection

In the thymus, the size of the DP cellular compartment seems largely determined by the input of competent DN cells (38). Thus, we assumed that on a TCRβ−/− background (precluding TCRβ-dependent DN-to-DP cell transition), numbers of DP Tcs homozygous for the Eβ169 mutation would be proportional to those of β-selected DN cells. Accordingly, the average of the number of DP cells in the Eβ169/169, TCRδ−/− vs WT thymi (0.2 vs 168) × 106, respectively (Table S1) estimated the production of TCRβ+...
DN Tcs in the mutant mice as ~0.1% that occurring in WT animals (a 1000-fold cutback), in general agreement with RT-PCR-assessed TCRα RNA expression data. Still, we stress that these figures may not reflect the full extent of loss due to this mutation when weighed against the Eβ intact element. Indeed, the near 2-fold reduction in Cc cellularity displayed by Eβ169/WT heterozygotes argues that, in this condition, the mutant allele/enhancer rarely contributed to the pool of TCRβ cells. Likewise, FACS and PCR analysis of LN Tcs from Eβ169/SJL compound mice pointed out that Eβ strongly overshadows Eβ169 in promoting cis recombination (Fig. S8, A–C, and data not shown; nota bene: the SJL spontaneous mutant strain carries an 80-kb deletion spanning from Vβ8.2 to Vβ8.1 (25), thus enabling the discrimination between Vβ169 micea

| Table I. TCRβ gene rearrangements in αβT cell hybridomas from Eβ169/169 micea |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Two VDJ Alleles | One VDJ Allele  |
| Eβ169 | 82 | 9 (11%) | 5 (6.1%) | 1 (1.2%) | 3 (3.7%) |
| Eβ169 | 62 (75.6%) | 3 (3.7%) | 8 (9.7%) |

a TCRβ T cell hybridomas (Hyb.) were tested for DJβ1-Jβ1, DJβ2-Jβ2, and DJβ2-Jβ2 rearrangements using total genomic DNA. For each class of hybridomas, the configuration of the remaining D-J clusters is indicated (GL, germline; DJ, DJβ rearranged). Numbers in parentheses indicate the percentages of the given subset relative to the total number of hybridomas.

Hybridomas carrying one DJβ1-Jβ1 rearrangement and the DJβ2-Jβ2 cluster in a germline configuration.

Among the eight hybridomas displaying a DJβ1-Jβ2 rearrangement, two also had a DJβ2-Jβ2 cluster in a germline configuration, indicating a Vβ-DJ1β rearrangement on the same allele; and six presented no other amplification, suggesting a Vβ-DJ2β rearrangement on the opposite allele.

Altered chromatin structure at Eβ169 alleles of DN T cells

The data above led us to postulate that Eβ169 may have a reduced capacity to induce chromatin cis remodeling and accessibility in T cell progenitors (see above). To investigate this, we compared epigenetic parameters between EβWT, Eβ169, and Eβ-deleted alleles backcrossed onto a Rag2-deficient background to ensure the germline configuration of TCRβ sequences while providing an enriched source of DN Tcs. To gain a general view, we analyzed the enrichment profiles of histone H3 lysine 4 dimethylation (H3K4me2; an epigenetic mark regularly associated with gene activation (39)) by ChIP-on-chip assays using ~150-bp tiled arrays comprising the entire TCRβ locus. As expected for a locus known to be expressed in DN Tcs, we observed H3K4me2 enrichment in the vicinity of most Vβ gene segments and along the DJβ-Jβ-Cβ regions at EβWT alleles. However, both Eβ169 and, to a larger extent, Eβ-deleted alleles demonstrated an overall decrease in H3K4me2 levels throughout the DJβ-Jβ-Cβ regions, even though, remarkably, the 5' and 3' Vβ regions showed no obvious effect (depicted in Fig. S9, A–C; all samples displayed similar H3K4me2 profiles at the Ephb6 gene 3' of the β locus and throughout the CD3e locus underlying the specificity of the observed β variations). A zoom on the 3' part of the TCRβ locus (Fig. 5) highlighted the average H3K4me2 patterns specific to each cis-regulatory context and showed the following: 1) compared with EβWT alleles, Eβ169 alleles displayed a global deficit in H3K4me2 that was most marked in and immediately around the DJβ1-Jβ1 cluster and, intriguingly, within both the Cβ1- and Cβ2-containing areas (however, the local distribution of H3K4me2 at the DJβ2-Jβ2 cluster appeared less dependent on enhancer input because it also almost reached WT levels at Eβ-deleted alleles); and 2) compared with Eβ-deleted alleles, the Eβ169 alleles displayed differential recovery of H3K4me2 patterns, spanning larger areas over the DJβ1-Jβ1 and DJβ2-Jβ2 clusters and, in an even more pronounced way, the enhancer 5’ flanking sequences. These observations were validated in conventional ChIP experiments assessing the levels (by quantitative PCR) of H3K4me2 at distinct sites within the DJβ-Jβ-Cβ regions (data not shown). The findings are consistent with a limited impact of Eβ169 on the relaxation of the chromatin template along the DJβ-Jβ-Cβ upstream clusters, which is massively reduced compared with the WT situation, yet moderately increased regarding the Eβ knockout. Focusing on the DJβ-Jβ regions in Rag-deficient or purified (cell sorted) DN Tcs, we observed additional evidence of epigenetic alterations at Eβ169 alleles that most likely contribute toward the impaired expression and recombination of these gene segments. Indeed, RT-PCR analysis of total RNA revealed severely reduced amounts of DJβ1- and, to a lesser extent, DJβ2-germline transcripts from the Eβ169 alleles (Fig. 6A; notice that germline transcription at Vβ gene segments mostly appeared enhancer independent, as previously suggested (16)). Also, bisulfite-assisted, quantitative investigations of CpG dinucleotide methylation in genomic DNA, a mark usually linked to gene suppression, revealed methylation at sites flanking the DJβ1 gene segment (including one such site within the DJβ1 3'RSS, the methylation of which impairs DJβ1 recombination (27)) in a high

![FIGURE 5. H3K4me2 chromosomal profiling at the 3' end of the TCRβ locus. The graphs depict Chip-on-chip data for H3K4me2 enrichment throughout the 3' region of the TCRβ locus in Tcs from Rag2-deficient (R−), R−Eβ169/169, and R−Eβ+ mice. Raw data were normalized using the overall signal in the entire microarray (excluding the probes within the TCRβ locus) and smoothed to a window of 1 kb in width.](http://www.jimmunol.org/)

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proportion of Eβ\textsuperscript{169} alleles, to levels only just lower than to those associated with Eβ-deleted alleles (Fig. 6B). We conclude that Eβ\textsuperscript{169} generally failed to induce proper chromatin remodeling throughout the cis-linked Dβ-β-Cβ clusters in DN cells. We suspect that this dysfunction, by reducing accessibility of the underlying sequences to the transcription and/or V(D)J recombination apparatus, lies at the heart of the severe block in the Dβ region. The diagram on the top depicts the position, relative to the first nucleotide in the Dβ1 gene segment, of the investigated CpGs. The triangles represent the Dβ1 5' and 3' RSSs. The lines on the left depict the methylation status of individual CpGs assessed via bisulfite treatment, cloning, and sequencing of single plasmid DNA prepared from R\textsuperscript{−/−}, Eβ\textsuperscript{−/−}, and Eβ\textsuperscript{169/169} DN Tcs, as indicated. Each line corresponds to a single DNA molecule, with circles representing the individual CpGs. ■ and □. Indicate methylated and unmethylated CpG sites, respectively. Averaged percentages of global methylation (analysis of the five CpG, in three separate experiments) are indicated. The graphs on the right give the percentages of methylation, assessed following bisulfite treatment and pyrosequencing, for the single Dβ1–3' RSS-located CpG, in genomic DNA purified from Rag2-deficient (left) or sorted DN (right) Tcs carrying Eβ (WT), Eβ\textsuperscript{169}, or Eβ-deleted (Eβ\textsuperscript{−}) TCRβ alleles, as indicated.

**Discussion**

We replaced the Eβ enhancer at the mouse TCRβ locus by a conserved Eβ\textsuperscript{169} fragment. This offered a unique opportunity to address the role of this core unit relative to the Eβ global activity. In assessing the Eβ\textsuperscript{169/169} mouse phenotype, we unveiled an unanticipated intricacy of enhancer functioning at the β locus, highlighting the value of studying regulatory elements at their cognate chromosomal location. Notably, our work emphasizes the importance of the Eβ binding sites in their entirety to achieve proper chromosomal structure and expression of the TCRβ gene, diversification of the Vβ repertoire, and production of physiological numbers of βT cells. Further observation revealed that on a per-cell basis, Eβ\textsuperscript{169} replacement results in a reduced early onset of locus expression and recombination, and increased heterogeneity in TCRβ mature gene expression, thereby shedding new light on models of enhancer function at Ag receptor loci.

**Efforts to define TCR gene enhancer cores**

For V(D)J recombination first relied on mouse transgenesis of reporter miniloci (e.g., Ref. 21, 40). However, positioned at their own chromosomal location, such reduced hubs mostly failed in sustaining recombination efficiently (41) (this study). More generally, even when promoting recombination within synthetic transgenes, TCR/Ig enhancers often do not work properly at heterologous sites (13, 41–44). The discordant results are unlikely due to a loss of enhancer function at knockin alleles caused by gene targeting. Specifically, the losP site left downstream of Eβ\textsuperscript{169} in this study after the removal of the selecting cassette (Fig. S3) has previously been used with no adverse effect on cis regulation in several knockin experiments at various loci, including the Eβ site at the TCRα locus (22). However, the chromosomal sites at which expressed transgenes tend to integrate (45) could be important, with successful expression depending on a permissive environment distinct from the regular situation. Another nonexclusive possibility could be that enhancer cores do not act efficiently over long distances. In transgenes, cooperating regulatory units (enhancer, promoter, etc.) lie a lot closer together than at their cognate loci. These rationales of proper chromatin landscape and of remoteness from actual target corroborate the validity of mutational studies on cis-acting elements in the native context.

**FIGURE 6.** A, RT-PCR analysis of germline transcription from the indicated TCRβ locus areas/gene segments in Tcs from Rag2-deficient (R\textsuperscript{−/−}), R\textsuperscript{−/−} Eβ\textsuperscript{169/169}, and R\textsuperscript{−/−} Eβ\textsuperscript{−/−} mice. The cDNA from the R\textsuperscript{−/−} cells was subjected to serial 10-fold dilutions before amplification. B, DNA methylation analysis of five CpG dinucleotides located in the Dβ1 region. The diagram on the top depicts the position, relative to the first nucleotide in the Dβ1 gene segment, of the investigated CpGs. The triangles represent the Dβ1 5' and 3' RSSs. The lines on the left depict the methylation status of individual CpGs assessed via bisulfite treatment, cloning, and sequencing of single plasmid DNA prepared from R\textsuperscript{−/−}, Eβ\textsuperscript{−/−}, and Eβ\textsuperscript{169/169} DN Tcs, as indicated. Each line corresponds to a single DNA molecule, with circles representing the individual CpGs. ■ and □. Indicate methylated and unmethylated CpG sites, respectively. Averaged percentages of global methylation (analysis of the five CpG, in three separate experiments) are indicated. The graphs on the right give the percentages of methylation, assessed following bisulfite treatment and pyrosequencing, for the single Dβ1–3' RSS-located CpG, in genomic DNA purified from Rag2-deficient (left) or sorted DN (right) Tcs carrying Eβ (WT), Eβ\textsuperscript{169}, or Eβ-deleted (Eβ\textsuperscript{−}) TCRβ alleles, as indicated.
within Eβ\textsuperscript{169}, with some displaying normal levels of binding (KLF and the E47 and HEB bHLH TFs), whereas others appeared to be poorly/incompletely recruited (the Runx1 and Ets1 TFs) (Fig. S1; S.S. and M.B., unpublished data). Cooperativity among TFs in binding to their cognate recognition sites in chromatin seems an important feature in the regulation of gene expression (48). The Eβ\textsuperscript{169} core alone might be unable to ensure such a cooperative binding, because surrounding sequences (and bound factors) perhaps critical in either promoting the availability of less accessible TFBSs or in favoring the formation and/or stabilization of an Eβ-nucleated, fully functional nucleoprotein scaffold(s) are missing. Such sequences may, for example, include the additional ETS/RUNX composite Eβ module (46, 49). In any case, our results using Eβ\textsuperscript{169} suggest that one critical pace in the activation of Eβ long-range functions may be the chromosomal accessibility of discrete TFBSs within the enhancer core. Future studies combining extensive footprinting and ChIP analyses should address this issue thoroughly.

It is generally difficult to draw conclusions from enhancer deletion experiments about the mode, whether it be graded or binary, of enhancer action (4). This is because of the mostly homogeneous, all-or-nothing expressed behavior of the related gene in enhancer-plus vs enhancer-minus cells. Instead, weakened enhancer activity, as is the case following the Eβ\textsuperscript{169} knockin technique, might expand differential cell expression, intensify phenotypic traits, and unmask the primary activation mode. Thus, in a framework in which TCRβ gene expression relies on enhancer inputs in both developing and mature T cells (13, 14, 20), Eβ\textsuperscript{169}-driven alleles yielded a reduced and variated TCRβ chain phenotype from the β-selection step onward, redolent of a graded conduct. In contrast, in earlier DN Tcs, Eβ\textsuperscript{169} appears to behave in an exceedingly stochastic fashion, engendering conditions for TCRβ gene recombination in a limited number of alleles only, more consistent with a binary mode of action. From this point of view, the data would predict a genetic system in which enhancer-regulated outcomes sequentially adopt a binary, then graded mode during T cell development. Such a dual behavior could be more prevalent in lymphoid gene regulation than initially thought because, for example, truncated versions of the IgH chain (IgH) gene enhancer EμL (which plays a significant role in the control mechanisms activating IgH gene assembly in pro-B cells) (50) also yielded variated expression of a IgH reporter gene inserted at a genomic site of a B cell hybridoma line (51). However, it should be noted that because enhancer-mediated activation of the TCRβ locus (in terms of histone posttranslational modification, germline transcription, etc.) could not be investigated (and linked to gene recombination) at the single-cell level in a Eβ\textsuperscript{169/169} situation, we are unable to eliminate the possibility that this enhancer works in a very inefficient graded mode during early T cell development.

How can these two distinct phenotypes be explained and what could be their physiological bearing on gene regulation? Regarding the first issue, it is relevant to recall that a binary transcriptional response becomes graded when the rate of promoter activation is increased, indicating that the experimentally observed binary and graded modes of gene regulation might arise from mere differences in transition rates between active versus inactive promoter/enhancer states (see Ref. 52 and references therein). Therefore, we propose two nonmutually exclusive options based on the observations that TCRβ gene expression generally increases as lymphoid T cells differentiate into more mature cell stages with, notably, transcription rates of VDJβ-assembled genes superceding those of germline Dβ-Jβ-configured loci (42): 1) a developmentally regulated increase in the enhancer intrinsic strength via the selection and/or stepwise assembly of a prevailing nucleoprotein scaffold (i.e., an enhancosome); and 2) the replacement of a DJβ-flanking promoter(s) by a stronger Vβ-associated promoter via Vβ-to-DJβ recombination. Whatever the exact mechanism of action, we underline in this study a potentially elaborate and particularly well-adapted behavior that might be adopted by the enhancer (and target promoters), allowing on the one hand a binary or all-or-none outcome in TCRβ gene expression that may be critical to cell fate decisions during DN early development, and, in contrast, graded alterations in more developed T cells that may permit the adaptation of a proportional response to selecting and/or activating signals.

The adaptation of regulatory elements, particularly transcriptional enhancers, has been considered as a possible major force in the evolution of complex genomic loci (53). In addition, recent evidence implies that enhancers are the most variable class of cis regulators between cell types and, as such, may be of primary importance in driving cell-type-specific patterns of gene expression (54). In these contexts, additional observations in Eβ\textsuperscript{169} knockin T cells may have original implications for our understanding of the evolutionary conserved control of TCRβ gene expression and, especially, V(DJ) recombination. Four aspects in particular draw our attention, as follows: 1) the finding of strictly monoallelic TCRβ gene rearrangements in a majority of the few developed Eβ\textsuperscript{169/169} T cells (Table I), perhaps implying a role for Eβ in adjusting a state of stochastic behavior of the TCRβ alleles in relation to the phenomenon of allelic exclusion (55); 2) the observation of selective DJ1β-Jβ rearrangements in those even scarcer T cells carrying dually rearranged VDJ/DJ TCRβ alleles, portending of a priority in enhancer cross-talk with this gene segment at the onset of TCRβ gene recombination; 3) the alteration of the Vβ repertoire via Vβ rearrangement even though germline transcription and H3K4me2 marks along the Vβ regions appeared enhancer independent, pointing to a potential role of Eβ in the V-to-DJ recombination phase (discussed further below); 4) the decreasing profile of H3K4me2 along the DJβ-β-Cβ regions possibly suggestive of cotranscriptionally targeting of histone posttranslational modifications also at these clusters (39, 56, 57). The Eβ\textsuperscript{169} mouse model may prove to be a valuable tool for future investigations aimed at further resolving these issues.

We observed a marked bias in the Vβ repertoire expressed by the peripheral αβ T cells from the Eβ\textsuperscript{169/169} mice, including, for example, a predominant representation of Vβ2, Vβ3.1/3.2, Vβ5.1,8.2. Vβ7, and Vβ14 (Fig. 3A). We can think of several molecular mechanisms and/or factors that could theoretically account for this surprising phenotype. First, the preferential selection of some particular VβDJβ-assembled units and their resulting products during T cell development, possibly linked to the paucity of DJβ joints in the Eβ\textsuperscript{169/169} mouse background, could explain this observation. Although the Vβ repertoire is not substantially altered during β-selection (35), it can be significantly shaped during αβ TCR selection of the DP Tcs in response to superantigens (see Ref. 58 and references therein), and recent evidence also favors the ability of DJβ complexes to influence TCRβ chain selection and peripheral Vβ repertoire even in the absence of such selecting molecules (59). Although the latter possibilities cannot be formally eliminated in the Eβ\textsuperscript{169/169} situation, it is, however, unlikely that they represent a unique explanation to the particular phenotype because, for several Vβ genes, we detected a biased representation at the level of Vβ-to-DJβ rearrangement in DN Tcs and/or of VβDJβ-assembled
TCRβ gene expression in both DN and DP cells (e.g., see Figs. 3E, 4, and S7). Second, in these contexts, it is formally possible that, relative to the Eβ WT element, Eβ169 differentially impacts the chromosomal accessibility of individual Vβ genes and/or their associated promoters. Again, we feel that this possibility is improbable based on the fact that we observed no significant alterations in either H3K4me2 enrichment levels (or/or their associated promoters. Again, we feel that this pos-

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Disclosures

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