Assessing the Role of the T Cell Receptor β Gene Enhancer in Regulating Coding Joint Formation during V(D)J Recombination*

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To assess the role of the T cell receptor (TCR) β gene enhancer (Eβ) in regulating the processing of VDJ recombinase-generated coding ends, we assayed TCRβ rearrangement of Eβ-deleted (ΔEβ) thymocytes in which cell death is inhibited via expression of a Bcl-2 transgene. Compared with ΔEβ, ΔEβ Bcl-2 thymocytes show a small accumulation of TCRβ standard recombination products, including coding ends, that involves the proximal DJβ-Jβ and Vβ14 loci but not the distal 5' Vβ genes. These effects are detectable in double negative pre-T cells, predominant in double positive pre-T cells, and correlate with regional changes in chromosomal structure during double negative-to-double positive differentiation. We propose that Eβ, by driving long range nucleoprotein interactions and the control of locus expression and chromatin structure, indirectly contributes to the stabilization of coding ends within the recombination processing complexes. The results also illustrate Eβ-dependent and -independent changes in chromosomal structure, suggesting distinct modes of regulation of TCRβ allelic exclusion depending on the position within the locus.

V(D)J recombination, one example of developmentally regulated DNA rearrangement known to occur in higher eukaryotes, is required for T cell receptor (TCR)β and Ig gene assembly and for T and B lymphocyte differentiation. This process is mediated by an enzymatic complex (the VDJ recombinase) whose targets (the recombination signal sequences or RSSs) flank dispersed V, D, and J gene segments and consist of conserved seven- and nine-nucleotide sequences (the heptamer and nonamer) separated by a non-conserved 12- or 23-nucleotide spacer (1). The recombination-activating-gene (RAG)-1 and -2 products constitute the core components of the recombinase (2). The RAG genes (possibly together with the original RSSs) are thought to have been transferred, in the form of a composite transposon, from the prokaryotic world to the germ-line of a common ancestor of the jawed vertebrates (3).

V(D)J recombination has been divided into two phases, based on in vitro recombination studies and the biochemical characterization of rearrangement products (4). In the first phase, the RAG factors (assisted by architectural factors; i.e., the high mobility group-1 and -2 proteins) initiate recombination by binding to, and introducing DNA cleavage at, two RSSs with spacers of dissimilar lengths. Ensuing DNA double strand breaks (DSBs) yield two pairs of products that consist of 5'-phosphorylated, blunt-ended RSSs (called signal ends, SEs), and the hairpinned, adjacent coding sequences (called coding ends, CEa). In a second phase, which depends on the coordinated action of the RAG and DNA repair non-homologous end-joining (NHEJ) factors (including Ku70/86, DNA-PK/Antemis, XRCC4, and DNA ligase IV), the two CEs are rapidly processed (this involves opening of the hairpins and, often, deletion and/or addition of nucleotides) and ligated to form a coding joint (CJ). With slower kinetics, the SEs are precisely joined to form a signal joint (SJ). As in the case of various recombination systems in prokaryotes (5), synaptic complexes of V(D)J recombination have been characterized that contain all or part of the aforementioned nucleotide sequences and catalytic factors (reviewed in Ref. 6).

V(D)J recombination is confined to immature lymphocytes because of the restricted expression of the RAG genes. In addition, it is tightly controlled with respect to lymphoid cell lineage and within a given lineage to the developmental stage and possibly also the TCR/ Ig allele used. For example, the TCRβ and TCRα genes (assembled from Vβ, DJβ, and Jβ and from Vα and Jα gene segments, respectively) are, with a few exceptions, rearranged exclusively in the T cell lineage, with TCRβ gene rearrangement in double negative (DN) pro-T cells preceding that of TCRα in double positive (DP) pre-T cells. Moreover, at the TCRβ locus, DJβ-to-Jβ rearrangement occurs first in CD44+CD25− DN thymocytes and, presumably, simultaneously on both alleles, followed by complete Vβ-to-DJβ assembly in more mature CD44hiCD25− cells, possibly with no allele synchronicity. Formation of a productive Vβ-to-DJβ joint (i.e. that maintains an open reading frame within the TCRβ
Enhancer-dependent Control of V(D)J Recombination

**Table I**

| Total | CD4⁺/CD8⁺ | CD4⁻/CD8⁻ | CD4⁻/CD8⁺ | CD4⁺/CD8⁻ |
|-------|------------|------------|------------|------------|
| wt (n = 4) | 208 × 10⁶ ± 28 | 6.4 × 10⁶ ± 1.8 | 179 × 10⁶ ± 20 | 18.7 × 10⁶ ± 4.01 | 4.8 × 10⁶ ± 2.56 |
| ΔEβ (n = 6) | 18.2 × 10⁶ ± 3.8 | 6.4 × 10⁶ ± 2.6 | 11.2 × 10⁶ ± 0.98 | 0.5 × 10⁶ ± 0.21 | 0.12 × 10⁶ ± 0.1 |
| ΔEβ B2 (n = 9) | 69.5 × 10⁶ ± 13.3 | 17.8 × 10⁶ ± 3.4 | 47.8 × 10⁶ ± 8.4 | 2.4 × 10⁶ ± 0.9 | 1.45 × 10⁶ ± 0.67 |

**RESULTS**

Production of the ΔEβ B2 Mouse and Characterization of Their TCR Gene Recombination Profile—A defect in resolving RAG-mediated DSBs that form at Eβ-deleted (Eβ⁻) alleles must result in cell death of the particular thymocytes. This could mask the actual levels of TCRβ recombination products (e.g., SEs) in ΔEβ thymi and the role played by Eβ in promoting RSS accessibility versus post-cleavage assembly in vivo. To overcome this problem, we analyzed TCRβ gene recombination at Eβ⁻ alleles in the situation where cell death is inhibited; ΔEβ mice were bred with mice that express an anti-apoptotic human Bcl-2 transgene (Tg Bcl-2) in T lineage cells (B2 mice). Cell counting and flow cytometric analysis indicated that constitutive Bcl-2 expression results in a slightly reduced proportion of DN cells in ΔEβ B2 versus ΔEβ thymi (from ~33 to 25.6%) and an increase in that of DP cells (from ~58 to 69%) although TCRβ⁻ and genuine single positive cells are still missing (Table I). Moreover, Bcl-2 expression was found to prolong cell survival of Eβ-deleted DN and DP thymocytes without rescuing the CD4⁰/CD25⁺ DN developmental block and accompanying cell proliferation defect (data not shown).

These findings are in agreement with those from earlier studies demonstrating that Tg Bcl-2 expression inhibits cell death without substituting for major selection processes in the developing T cells such as pre-TCR-based β-selection or TCRαβ-positive selection (17, 20–22).

We started our evaluation of TCRβ gene rearrangement in Tg Bcl-2-expressing thymocytes using well defined, semi-quantitative PCR assays to test for SE, SJ, and CJ products at the Dβ2-Jβ2 cluster (Fig. 1A). In agreement with earlier findings (16), both 3’ Dβ2 SEs and Dβ2-to-Jβ2 SJs were detected in ΔEβ thymocytes, at decreased levels compared with the wt

**EXPERIMENTAL PROCEDURES**

Mice—Single knockout, RAG-1-deficient (Rag), Eβ-deleted (ΔEβ), and double knockout (Rag ΔEβ) mice, as well as mouse housing and analyzing conditions, were described previously (16), wt C57BL/6J and CB17 SCID (Scid) mice, transgenic Eβ-Bcl-2 (B2) and P14 TCRβ/V8.1-DJβ2.4 (p14) mice (17, 18), and combinatorial knockout and transgenic (ΔEβ B2) mice were handled similarly.

Thymocyte Preparation and Cell Culture Conditions—Thymocyte preparation and cell culture have been described previously (15). 150 μg of anti-CD3-ε (2C11; Pharmingen) monoclonal antibody were utilized for intraperitoneal injection of 4-week-old animals.

Flow Cytofluorometry Analyses and Cell Sorting—Cell-staining conditions, flow cytometric analyses, and cell purification by cell sorting were carried out as described by Leduc et al. (14).

Molecular Analyses of V(D)J Recombination Products and Chromatin Structure at the TCRβ Locus—Nucleic acid extraction, assays for SE, SJ, CJ, and hybrid joints (HJ) products, sequencing, and RT-PCR analyses, as well as ligation-mediated (LM)-PCR analysis of restriction enzyme accessibility and chromatin immunoprecipitation (ChIP)-PCR analysis of histone H3 acetylation were performed as described previously (13, 15, 16). Assays for CE products were performed according to Zhu et al. (19), with PCR products for CE/SE/SEs being separated through polyacrylamide gels (instead of agarose gels, as for the analysis of the amplified products in all other PCR assays). All PCR experiments were performed at least twice with consistent results. A list of oligonucleotide primers used in these experiments is available upon request.

In the mouse germline, the ~500-kb TCRβ locus consists of ~35 distinct Vβ genes that, for the most part, are spread over a large DNA region extending from 200–450 kb upstream of the duplicated Dβ1-Jβ1-β1(Dβ2-Jβ2-β2 clusters, except for one (Vβ14), which lies in opposite orientation. ~10 kb downstream (10). A single TCRβ gene enhancer (Eβ) has been described, which is located within the Cβ2-β14 intervening region (11). Targeted deletion of Eβ has revealed a striking phenotype.

In the T cell lineage, Dβ-to-Jβ CJs are drastically reduced at the targeted TCRβ allele(s) (>50–100-fold compared with the wild-type (wt)), with an even more severe defect in Vβ-to-DJβ CJs. In homozgyously deleted (Eβ⁻⁻) thymi, no TCRβ chains are made, and no αβ T cells can develop (12–14). Moreover, comparative analysis of molecular markers for chromatin structure in developmentally arrested DN, CD25⁺ pro-T cells from either Rag⁻⁻ (hereafter Rag) or combinatorial (Rag⁻⁻ × ΔEβ; Rag ΔEβ) mice provided compelling evidence for a primary function of Eβ in regulating chromatin opening within a limited (~25 kb) upstream domain comprised of the Dβ-Jβ-Cβ clusters, with a minor effect on the 5’ distal Vβ genes or 3’ proximal Vβ14 (15). However, RAG-mediated SEs at Dβ and Jβ gene segments (as well as the corresponding SJs) can readily be detected at Eβ-deleted alleles, although at a level 10–30-fold lower compared with the wt (16). The facts that TCRβ rearrangement was initiated in ΔEβ thymocytes, but that formation of CJs may be more severely impaired, suggested an additional function for Eβ in CE processing. Here, using ΔEβ mice expressing an anti-apoptotic Bcl-2 transgene, we attempt to better delineate the actual impact of Eβ in enhancing DNA repair/CJ formation during TCRβ locus recombination, relative to its effects on chromosomal accessibility.

Our findings are consistent with a model in which Eβ impinges on the stabilization of CE within the post-cleavage synaptic complex, in addition to its primary functions in regulating chromosomal access and locus expression.
Oligonucleotide primers and the linker used in LM-PCR are schematized by shaded horizontal arrows and asymmetric pairs of bold lines. A, genomic DNA from thymocytes of the indicated mice (including two Bcl-2 individuals) was analyzed by LM-PCR for SEs 3′ of Dβ2 (ΔEβ SE). Lanes 1–4, serial dilution analysis using linker-ligated DNA from a wt thymus (lanes 1, undiluted thymus; lanes 2 and 3, thymus/kidney, 1/5 and 1/25 dilutions; lane 4, undiluted kidney). PCR amplifications for a ΔEβ-containing DNA fragment (Cβ) were carried out in parallel to control for sample loading. C, DNA samples were analyzed by PCR for Dβ2-to-Jβ2.1 SJs (ΔEβ SJ). Digestion of amplified DNAs by the restriction enzyme ApaLI (arrows) was performed to check the accuracy of RSS ligation to form the SJs. D, PCR analysis of Dβ2-to-Jβ2.1/2.6 CJs (ΔEβ CJ; top panels) and control Cβ amplifications (Cβ; bottom panels). GI, Dβ2/Jβ2-containing germline fragment. The asterisk indicates a nonspecific band that was also amplified from kidney DNA. Serial dilution was as in B except that genomic (non-ligated) DNAs were used; dilution in lanes 2 and 3 was 1/4 and 1/8, respectively. E, quantitation of SE, SJ, and CJ products. PhosphorImager signals for the recombination products were quantified by densitometric scanning and corrected according to the signal from the Cβ control. The corresponding signals in wt thymocytes.

Fig. 1. Dβ2-to-Jβ2 SE, SJ, and CJ products in ΔEβ B2 thymocytes. A, schematic representation of the PCR assays used to analyze SE, SJ, and CJ products within the Dβ2-Jβ2 gene cluster. RSSs of 23- or 12-nucleotide spacer are figured by shaded horizontal arrows. Oligonucleotide primers and the linker used in LM-PCR are schematized by shaded horizontal arrows and asymmetric pairs of bold lines. B, genomic DNA from thymocytes of the indicated mice (including two ΔEβ B2 individuals) was analyzed by LM-PCR for SEs 3′ of Dβ2 (ΔEβ SE). Lanes 1–4, serial dilution analysis using linker-ligated DNA from a wt thymus and/or kidney (lane 1, undiluted thymus; lanes 2 and 3, thymus/kidney, 1/5 and 1/25 dilutions; lane 4, undiluted kidney). PCR amplifications for a ΔEβ-containing DNA fragment (Cβ) were carried out in parallel to control for sample loading. C, DNA samples were analyzed by PCR for Dβ2-to-Jβ2.1 SJs (ΔEβ SJ). Digestion of amplified DNAs by the restriction enzyme ApaLI (arrows) was performed to check the accuracy of RSS ligation to form the SJs. D, PCR analysis of Dβ2-to-Jβ2.1/2.6 CJs (ΔEβ CJ; top panels) and control Cβ amplifications (Cβ; bottom panels). GI, Dβ2/Jβ2-containing germline fragment. The asterisk indicates a nonspecific band that was also amplified from kidney DNA. Serial dilution was as in B except that genomic (non-ligated) DNAs were used; dilution in lanes 2 and 3 was 1/4 and 1/8, respectively. E, quantitation of SE, SJ, and CJ products. PhosphorImager signals for the recombination products were quantified by densitometric scanning and corrected according to the signal from the Cβ control. Graphic representation for each product is shown, relative to a 100% value as defined from the corresponding signals in wt thymocytes.

(See Fig. 1, B (lanes 1–5) and C, lanes 1–12), whereas Dβ2-to-Jβ2.1/Jβ2.6 CJs appeared to be more severely reduced (Fig. 1D, lanes 1–5). According to PhosphorImager scanning and densitometric analysis (Fig. 1E), SEs and SJs without Eβ were reduced to 16–20% of those in wt thymi whereas CJs were reduced to 7%, arguing that Eβ deletion indeed impacts on Dβ2/Jβ2 RSS cleavage with an additional, weaker effect on CE resolution. Significantly, all the three types of recombination products were amplified at increased levels in ΔEβ B2 compared with ΔEβ thymocytes, including Dβ2-to-Jβ2 CJs (Fig. 1, B, C, and D, lanes 5–7, 9–20, and 5–7, respectively). By densitometric comparison of ΔEβ and ΔEβ B2 thymocytes (Fig. 1E), we estimated that SEs and SJs increased from, respectively, 16 to 29% (1.18×) and 20 to 26% (1.33×) relative to those in wt cells, whereas CJs showed a greater rescue, from 7 to 30% (4.33×). Overall, the effect of Bcl-2 in rescuing Dβ2-to-Jβ2 CJs may thus result from a small accumulation of intermediate SE/CE products combined to a specific, additional enhancement of CE processing (formally, because the CJ effect should be the product of CE/CE formation or appearance) and CE resolution, the apparent effect on CE resolution in this case could be 2.4× (4.3/1.8) only. Consistent with this, the germline fragment containing Dβ2/Jβ2 gene segments was detected at high levels in both ΔEβ and ΔEβ B2 but not wt thymocytes (Fig. 1D). Also, as judged from similar SE and CJ assays, recombinase activity in ΔEβ B2 cells generally appears less marked within the Dβ1-Jβ1 cluster compared with Dβ2-Jβ2 (data not shown).

The small accumulation of Dβ2-to-Jβ2 recombination products in ΔEβ B2 thymocytes prompted us to also check for the presence of Vβ-to-DJβ2 CJs. Surprisingly, Vβ14-to-DJβ2 CJs were found in thymocytes from the ΔEβ B2 mice (at levels varying from 12 to 48% of those in the wt) whereas these products were routinely not detected in cells from ΔEβ littermates (e.g., Fig. 2). Analysis of SEs at Vβ14 once again argued for a specific effect of Bcl-2 expression on the rescue of CEs compared with SEs (see below). However, Vβ-to-DJβ2 assembly in ΔEβ B2 thymocytes seems to be restricted to Vβ14 only (i.e., the single Vβ gene located on the 3′ end of the TCRβ locus), as no accumulation of CJs was detected for several 5′ Vβs, including members located in the proximal (Vβ18), median (Vβ20, Vβ11, and Vβ5), or distal (Vβ4) parts of the 5′ Vβ gene cluster (Fig. 2) (data not shown).

Sequence analysis of Dβ2-to-Jβ2.5/2.6 CJs from ΔEβ B2 thymocytes generally showed the hallmarks of normal CE processing prior to joining, including occasional P and/or N nucleotide additions and short deletions; one Dβ2-to-Jβ2.5 CJ showed an unusually long (11-bp) N region (data not shown) (also, in Fig. 1C, SE resolution in ΔEβ B2 T cells appears to mostly result in standard SJs as they were digested by restriction enzyme ApaLI that cleaves the perfect fusion of two hep-
mung bean nuclease (to open the hairpin structures) and T4 could not be detected by LM-PCR of genomic DNA treated with mocytes from the /H9004 cated proximal to the E/H9252 Imager signals from each amplified fragment, yielding 48% (thymocytes was performed by densitometric scanning of Phosphor-

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that there is no accumulation of CEs at Eβ alleles, even under the condition of constitutive Bcl-2 expression. 

HJs are non-standard V(D)J rearranged products that result from the attack of a hairpinned CE by the SE liberated from the opposite gene segment participating to the recombination complex (schematized in Fig. 3). The reaction, which is mechanistically similar to a transposition, was partially reproduced in vitro using purified, truncated forms of RAG proteins (catalytically active core RAGs), in the absence of the NHEJ factors (23). In vivo, HJs are found at a low level in wt lymphocytes. In line with the in vitro results, it is widely accepted that HJs predominate in developing lymphocytes with NHEJ deficiencies. However, a recent study (24) suggests a more complex situation, as the non-core regions in full-length RAGs seem to down-modulate HJ levels in the absence of NHEJ. Intriguingly, HJs involving CEs 3' of Dj2 and Jβ2 SEs could not be detected in ΔEβ or ΔEβ B2 thymocytes (Fig. 3, bottom panels). Thus, Tg Bcl-2 expression in Eβ− thymocytes results in an accumulation of SE/SJ/CJ products of Dj8-to-β rearrangement, but it has a negligible impact on parallel accumulation of CEs and HJs. Overall, these data do not support a role for Eβ in the recruitment of NHEJ factors to the recombination complex (see “Discussion”). However, they do not exclude an indirect effect of Eβ on CJ formation; e.g. the stabilization of CE intermediates within the PCS complex.

TCRβ Gene Expression in Eβ-Deleted Thymocytes—At TCR/Ig loci, activation of regional transcription and V(D)J recombination frequently (but not always) correlate (4). Earlier studies have shown that transcription of the unrearranged (germline) Dβ-Jβ loci is strongly inhibited at Eβ− compared with Eβ+ alleles in early developing T cells, whereas that of Vβ genes is not significantly altered (15). We have analyzed TCRβ gene expression in ΔEβ B2 versus ΔEβ thymocytes, using RT-PCR assays to study transcription through either germline Jβ or Vβ gene segments (Jβ Gl or Vβ Gl) or through partially (D(J)b) or completely (VβD(J)b) re-arranged products (Djβ Rg or Vβ Rg). We found no Jβ Gl

\* W. M. Hempel and N. Mathieu, unpublished results.
transcription in ΔEβ B2 thymocytes at the Δβ1-Jβ1 or Δβ2-Jβ2 loci; also, ΔJβ Rag transcription was negative in these cells (Fig. 4A, upper two panels) (data not shown). Therefore, despite the evidence of Δβ-to-Jβ recombination at Eβ⁺-alleles, there is no evidence of transcription through these loci (including in the Tg Bcl-2 expressing cells). This is yet another example of differential activation of the two processes. In addition, we found Vβ Gl transcription for Vβ5, Vβ11, and Vβ14 at Eβ⁺-alleles, but no Vβ Rag transcription, including for Vβ14 and the ΔEβ B2 thymocytes (Fig. 4A) (data not shown), in agreement with the lack of TCRβ⁺ cells (and β-selection) in the ΔEβ/ΔEβ mice, as evidenced by flow cytometry. Whereas Vβ Gl transcription of Vβ5 (and Vβ11) appeared to be reduced in ΔEβ B2 compared with ΔEβ thymocytes, that of Vβ14 was unchanged (or increased slightly; e.g., see Fig. 4A). These differential profiles likely result from a reduced DN/DP cell ratio in ΔEβ B2 versus ΔEβ thymi, coupled with Eβ-independent developmental changes in Vβ Gl transcription (rather than from intrinsic differences between the two types of ΔEβ B2 and ΔEβ cells). Indeed, we found a dramatic down-regulation of Vβ5 and Vβ11 Gl transcripts in purified DP versus DN ΔEβ B2 thymocytes and steady-state (or slightly increased) levels of Vβ14 Gl transcripts (Fig. 4B) (data not shown). Therefore, Vβ Gl transcription profiles in DP ΔEβ B2 cells (5' Vβ Gl / Vβ14 Gl) correlate with those of Vβ-to-Jβ DJ 6 SJs in ΔEβ B2 thymi. This lead us to investigate whether, in this situation, TCRβ recombination also depends on DN-to-DP development and, potentially, Eβ-independent changes in chromosomal access.

Developmentally Regulated Activity of the VDJ Recombinase May Be Compromised at Eβ-Deleted Alleles—To investigate whether the predominance of TCRβ SE, SJ, and CJ products in ΔEβ B2 versus ΔEβ thymi also correlates with their differences in cell distribution, we purified DN and DP thymocytes from both types of mice and analyzed the rearrangement of their TCRβ locus, as described above. We first focused on ΔD2-to-Jβ2 rearrangement. Remarkably, we found SEs' of ΔD2 and ΔD2-to-Jβ2-6 SJs predominantly in DP thymocytes from both ΔEβ and ΔEβ B2 mice and at higher levels in Tg Bcl-2 expressing cells; however, the effect of Bcl-2 on the accumulation of rearrangement products was also visible in DN pro-T cells (Fig. 5, A (lanes 3–6) and B (lanes 5–12)). Likewise, in Eβ-deleted animals, we detected ΔD2-to-Jβ2 CJs predominantly in ΔEβ B2 DP thymocytes; Bcl-2 also had an effect on CJ accumulation in DN cells (Fig. 5C). Notably, CJs in ΔEβ B2 DN cells were detected at a slightly higher level (~1.2×) compared with those in ΔEβ DP cells despite a bias for SEs (~2×) in favor of the latter (Fig. 5, C and A, lanes 4 and 5, respectively) (data not shown). Further quantitation analysis (Fig. 5D) indicated that SEs without Eβ are reduced to ~7% (DN) and ~26% (DP) of those in the corresponding wt cells, whereas CJs are reduced to ~4% (DN) and ~10% (DP). By comparison, in ΔEβ B2 thymocytes, SEs increased slightly to, respectively, ~12% (DN; ~1.7×) and ~42% (DP; ~1.6×) whereas CJs increased to 24% (DN; ~6×) and ~36% (DP; ~3.6×). These data confirm our previous results of a preferential, although limited effect of Bcl-2 on ΔDβ Jβ CE resolution at Eβ⁺ alleles, which is apparent in both DN (~3.5× (6/1.7)) and DP (~2.2× (3.6/1.6)) cells. They further suggest that, in most Eβ-deleted thymocytes, recombination activity at the ΔDβ RSSs is extended/delayed to cells that have developed to the DP stage. Significantly, we also find high levels of SEs' of ΔD2 and ΔD2-to-Jβ2-6 SJs in
both DN and DP wt thymocytes (Fig. 5, A (lanes 1 and 2) and B (lanes 1–4), in agreement with similar findings at the Dj1-Jj1 gene cluster (25). Because unresolved DSBs generated in DN cells would arrest cell proliferation during DN-to-DP cell differentiation, the detection of SEs 3’ of Dj in DP cells from wt mice must reflect cell autonomous activity of the RAG factors at unarranged DβJβ loci (25) and/or at Dj to Jβ SJs within extrachromosomal circles (26).

**Eβ-Independent Changes in Chromatin Structure at the TCRβ Locus during T Cell Development**—The extended lifespan conferred by a Bcl-2 transgene is likely to provide an extended time window per cell for V(D)J recombination (27), thus accounting for one aspect of our results (i.e. the rescue of CJ formation; see below). However, other mechanisms must account for the accumulation of SEs on a large scale in DP cells (including DP cells that develop in the absence of B-selection) (14) and for the profile of Vβ gene recombination in ΔEβ B2 thymocytes. One possibility would be that, upon DP development, accessibility to the RAG factors is established along the DβJβ loci in an Eβ-independent manner, whereas a repressive structure invades the 5’ Vβ genes but not Vβ14. This would impact on the controls of both TCRβ gene recombination and allelic exclusion during T cell differentiation.

We have tested this model and analyzed chromatin structure at discrete TCRβ regions using nuclei from Rag and Rag ΔEβ thymocytes (Eβ+ and Eβ+ DN cells, respectively) and enzyme restriction/LM-PCR chromosomal accessibility assays, as described previously (15). Thymocytes from Rag and Rag ΔEβ mice treated by intraperitoneal injection of anti-CD3-ε monoclonal antibody (to mimic pre-TCR signaling) (28) were used as a source of DP-enriched nuclei. Fibroblasts were used as a non-lymphoid control. As predicted, we found that the Jj1 region is more likely to be cleaved in Eβ+ DP (Rag ΔEβ CD3) and in Eβ+ DN (Rag) or DP (Rag CD3) thymic nuclei, compared with Eβ+ DN (Rag ΔEβ) thymic or to fibroblastic nuclei (Fig. 6A, top panels; consistent results were also obtained at the Jβ2 locus). Furthermore, we found Vβ5 to be more resistant to cleavage in both Eβ+ and Eβ+ DP (Rag CD3 and Rag ΔEβ CD3) nuclei relative to Eβ+ or Eβ+ DN (Rag and Rag ΔEβ) nuclei (Fig. 6A, middle upper panels; similar results were found at Vβ11 (data not shown). Finally, we found Vβ14 to be cleaved in T cell nuclei, independent of the developmental (DN or DP) stage and the presence of Eβ (Fig. 6A, middle lower panels).

Histone acetylation has emerged as an important regulator of chromatin structure (29) and of locus accessibility for V(D)J recombination in vivo (30). We used ChIP-PCR to compare histone H3 acetylation at Dj-Jj and Vβ loci in Rag ΔEβ CD3 (DP) versus Rag ΔEβ (DN) thymocytes. We found that, during the course of anti-CD3-ε-induced DN-to-DP differentiation, H3 acetylation of Eβ− thymocytes (i) increases slightly at βJ and Jβ1 and more readily, at Jβ2; (ii) decreases (by ~2-fold) at Vβ5; and (iii) is maintained at a steady state level at Vβ14 (Fig. 6B). Overall, these data support our model of Eβ-independent, DN-to-DP regulated changes in chromosomal organization (including histone H3 acetylation) at distinct regions throughout the TCRβ locus.

**Eβ-Independent DSB Cleavage at Vβ14 in DP Thymocytes**—The drop in accessibility of 5’ Vβ genes in CD3-ε triggered Rag thymocytes likely mimics a physiological mechanism involved in the feedback inhibition of Vβ gene rearrangement in response to pre-TCR-induced signaling (i.e. allelic exclusion). In this context, persistent accessibility of the Dβ-βJ and Vβ14 loci potentially threatens allelic exclusion so that this process must be regulated differently at the 3’ end of the TCRβ locus. The finding of Vβ14-to-DJβ3 SJs in ΔEβ B2 thymocytes also raises the question as to whether Eβ+, in conjunction with cell death control, could participate in this regulation. To address these issues, we tested Eβ− and Eβ− thymocytes for the presence of SEs at both Vβ5 and Vβ14 and of Vβ5-Vβ14-to-DJβ2 CJs. As a source of Eβ+ or Eβ− thymocytes, we used wt
mice and TCRβ transgenic mice (p14, a model for TCRβ allelic exclusion (18)) or the ΔEβ and ΔEβ B2 mice, respectively.

As expected, we detected SEs at Vβ5 predominantly in DN thymocytes from the Eβ− animals and at a reduced level in p14 compared with wt cells (a >6-fold decrease as judged from densitometric analysis of PhosphorImager signals) whereas, in agreement with previous findings, these products were hardly visible in Eβ− (ΔEβ and ΔEβ B2) thymocytes (Fig. 7A, top panel). In contrast, we found Vβ14 SEs to predominate in DP cells from the p14, ΔEβ, and ΔEβ B2 mice (Fig. 7A, middle panel; Vβ14 SEs were occasionally detected, at a lower level, in DN and/or DP cells from wt mice) (data not shown). Yet both Vβ5 and Vβ14 CJs were normally found in wt thymocytes and were strongly reduced in p14 cells (although, possibly, to a lesser extend for the Vβ14 CJs); as expected, CJs were not detected at Eβ− alleles except for Vβ14 and the ΔEβ B2 cells (Fig. 7B). The latter findings, coupled to those of Vβ14 SEs in ΔEβ DP thymocytes (Fig. 7A, lane 7), are consistent with a specific effect of Bcl-2 on CE processing also at Vβ14. Two other elements should also be considered. First, a 6-fold decrease of Vβ5 SEs between wt and p14 DN cells (LM-PCR assays of Fig. 7A, lanes 2 and 4) can account for the drop of the corresponding CJs (PCR assays of Fig. 7B, lanes 4 and 6; also see lanes 2 and 3), implying that exclusion of Vβ5 rearrangement is likely to be regulated primarily at the level of chromosomal access. In p14 thymocytes, Vβ5 SEs may correspond to normally rearranging alleles; e.g., in a few DN cells that do not express the β transgene. We cannot exclude, however, that inhibition of Vβ5 rearrangement is regulated beyond the step of DSB cleavage in a small population of TCRβ rearranging cells. Second, Vβ5 and Vβ14 CJs look similar in wt thymocytes (Fig. 7B, lanes 4 and 5). It is thus reasonable to assume that, similar to Vβ5, most of the Vβ14 CJs detected in wt DP cells are indeed generated in DN cells and then expanded by β-selection. The failure to detect Vβ14 SEs in wt DN thymocytes may be because of a specific feature(s) in the processing of these products, linked to the mode of Vβ14 rearrangement by DNA inversion and the constraint to preserve chromosome integrity at the site of SJ formation (31). Conversely, increased accumulation of Vβ14 SEs in DP thymocytes from p14 and Eβ-deleted mice may reveal a disorder of the latter control in these cells and, indeed, a unique mode of allelic exclusion at the 3′ end of the TCRβ locus, evidenced here by the high frequency of attempted Vβ14 rearrangement. Given the accessibility of the Vβ14/DJβ loci, the level at which Vβ14 23-nucleotide RSS cleavage can be observed in total DP cells is predicted to depend on the proportion of complementary 5′ DJβ 12-nucleotide RSS left available for synapsis (4, 32, 33).

Processing of Vβ14 SEs into CJs at Eβ− alleles can be rescued, to some extent, by constitutive Bcl-2 expression. To check whether this also occurs in the presence of Eβ−, we analyzed thymocytes from p14 B2 double transgenic mice. Indeed, we found increased levels (~2.9×) of Vβ14 CJs in p14 B2 compared with p14 thymocytes, whereas Vβ14 SEs were roughly equivalent in DP cells from both types of mice (Fig. 7C). Altogether, the above data strongly suggest that exclusion of Vβ14 rearrangement proceeds through a unique mechanism, one aspect of which could be a specific defect in the resolution of discrete DNA DSBs and, most likely, the induction of cell death. Eβ does not appear to interfere with these processes, including the initial steps of the recombination reaction (synapsis and RAG-mediated DNA cleavage) during attempted Vβ14-to-DJβ rearrangement.

**Fig. 7.** Analysis of Vβ SE and CJ products in DN and DP thymocytes from Eβ− and Eβ− mice. A and C, lower panels, genomic DNA from total and/or DN- and DP-sorted thymocytes in the indicated mouse lines was analyzed by LM-PCR for the presence of Vβ5 or Vβ14 SEs (Vβ5 SE and Vβ14 SE, respectively) and by long range PCR for the presence of Vβ5-(D)Jβ2 or Vβ14-(D)Jβ2 CJs (Vβ5 CJ and Vβ14 CJ, respectively; B and C, upper panels). In A, thymus DNA from a Rag mouse was used as a negative control. Serial dilution analyses of wt thymus DNA shown in B and C were as in Fig. 1D. C, PhosphorImager scanning analysis of recombination products in p14 B2 versus p14 thymocytes gave the following results (after normalization to Cβ controls): CJs, 2.53/0.88; SEs, 2.2/1.8, respectively. Because control Cβ2 amplifications (Cβ) used oligonucleotide primers located, respectively, upstream of (5′ primer) and within (3′ primer) exon 1 of Cβ2, the p14 transgene was not detected in this assay.

**DISCUSSION**

**A Critical Function for Eβ in Promoting Access to the DJβ-Jβ Domains in pro-T Cells**—We have analyzed TCRβ gene rearrangement in ΔEβ B2 thymocytes to better assess Eβ function in the regulation of CJ formation during V(D)J recombination. Compared with ΔEβ, ΔEβ B2 thymocytes show a small accumulation of TCRβ standard recombination products, most notably CJs, that is confined to proximal DJβ-Jβ and Vβ14 loci. Although detectable in DN CD25− pro-T cells, these effects predominate in cells that have differentiated to the DP stage.
Enhancer-dependent Control of V(D)J Recombination

Developmental cell selection is unlikely to account for these findings, as no TCRβ rearranged products could be detected at either the RNA or protein levels. Instead, evidence for VDJ recombination activity at these loci also in Eβ– DP thymocytes, and the fact that the rearrangement profiles at distinct TCRβ loci in DN and DP ΔEβ thymocytes correlate with their level of chromosomal accessibility in the particular cell subset, argue for delayed access of the recombinase to Eβ– alleles. Besides pointing to a possible, likely indirect effect of Eβ in assisting CJ formation, these data most notably emphasize this critical function of the enhancer for chromatin opening within the DJ-β-Jβ domains, which, unexpectedly, appears also limited to an early window of T cell development only (i.e. anterior to the CD44+/CD25+ DN cell stage).

A Mechanism of Enhancement of DJβ-to-βJ CJ Formation by Eβ—Resolution of Rag-mediated DNA breaks that can happen in Eβ-deleted thymocytes is improved by Tg Bcl-2 expression, apparently with a slightly greater impact on CJ compared with SJ formation (e.g. Fig. 2). May this tell us something about a putative role of Eβ in enhancing CE processing? First, the effects conferred by Bcl-2 transgenes on developmental processes in lymphoid cells (including VDJ recombination) have generally been attributed to cell extended lifespan (27, 34), although a non-conventional role of Bcl2 and incidental effect(s) on the processing of injured DNA cannot be formally ruled out (35). Second, the resolution of CE and SE products is thought to proceed along two different pathways, involving distinct requirements and kinetics (4). Thus, although a deficiency in any of the factors of the NHEJ apparatus results in a CJ defect, some proceed with unaltered SJ formation (e.g. the SCID defect resulting from DNA-PK deficiency). Also, whereas SEs accumulate in lymphoid cells undergoing VDJ recombination (and are eventually resolved after Rag expression is down-regulated), CEs are difficult to detect in the wild-type situation (they accumulate in DNA-PK-deficient lymphocytes), indicating that CJ formation must be tightly linked to RSS cleavage. In possible relation to these distinct behaviors, in vitro studies have suggested diametrically opposed stability of VDJ recombination post-cleavage complexes depending on product content; complexes consisting of the two CEs and two SEs appear to be highly unstable whereas those consisting of the two SEs bound by the Rag factors are resistant to dissociation challenges (reviewed in Ref. 6). We believe that a direct effect of Eβ in mediating the recruitment of DNA-PK (or a DNA-PK/Artemis complex (36)) is unlikely, based on the CJ formation and cleavage rates (Fig. 2). May this tell us something about a regulatory function of the enhancer for chromatin opening within the DJ-β-Jβ domains, which, unexpectedly, appears also limited to an early window of T cell development only (i.e. anterior to the CD44+/CD25+ DN cell stage).

In DP thymocytes, the 3′ end of the TCRβ locus containing the DJ-Jβ-Cβ and Vβ14 domains maintains (or gains in the case of Eβ– alleles and the DJβ-Jβ-Cβ clusters) chromosomal accessibility. This might involve the regulated activation of additional cis elements that normally act redundantly with Eβ within these loci (40). At this stage of development and in the wild-type situation, fully derepressed chromatin may be required to ensure high levels of expression of a rearranged VβDJβ-Cβ unit. The ensuing drawback is cleavage by the RAG machinery past the β-selection checkpoint, with consequences such as the specific accumulation of DJβ and Vβ14 SEs and DJβ CJs in DP thymocytes (see Fig. 5, A and C and see Fig. 7A) (25). Nevertheless, the fact that levels of Vβ14 CJs remain extremely low in p14 DP cells (Fig. 7B) indicates that these consequences on TCRβ allelic exclusion are minimized, possibly involving regulated cell death (Fig. 7C). Recent results (47) suggest that programmed cell death may be a parameter that also limits ongoing rearrangements along the TCRα locus in DP thymocytes. In this context, the particular situation of Vβ14 and inversional mode of rearrangement could concur to the surprisingly opposite outcomes of Vβ14-to-DJβ versus DJβ-
to-Jβ attempted recombination in DP cells. Notably, intrachromosomal Vβ14 to-DJκ SJs within this accessible part of the locus could be ideal targets for DSB formation by a RAG-mediated nick-nick mechanism (26). It is, however, important to stress that the actual levels of V(D)J recombination at Dβ locus could be ideal targets for DSB formation by a RAG-mediated recombination (27). We searched for TCR β diversity using FISH analysis and PCR assays (including assays to search for}