Differential Activation of Transcription versus Recombination of Transgenic T Cell Receptor β Variable Region Gene Segments in B and T Lineage Cells

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Summary

We have tested the ability of the T cell receptor β (TCR-β) transcriptional enhancer (E_β) to confer transcriptional activation and tissue-specific V(D)J recombination of TCR-β V, D, and J segments in a transgenic minilocus recombination substrate. We find that the minimal E_β element, as previously shown for a DNA segment that contained the E_μ element, promotes a high level of substrate D to J_β rearrangement in both B and T cells, but only promotes V_β to DJ_β rearrangement in T cells. Thus, both the E_μ and E_β elements similarly direct V(D)J recombination of this substrate in vivo, supporting a general role for transcriptional enhancers in the normal regulation of this rearrangement process. Surprisingly, however, we found that both the V_β and DJ_β portion of the constructs were transcribed in an enhancer-dependent fashion (conferred by either E_μ or E_β) in both B and T lineage cells, including normal precursor B cells propagated in culture. These findings indicate that, at least in some contexts, transcriptional activation, per se, is not sufficient to confer V(D)J recombinational accessibility to a substrate V gene segment.

The genes that encode the variable regions of Ig and TCRs are assembled from germline V, D, and J gene segments by a process referred to as V(D)J recombination (1). Both Ig and TCR variable region gene segments are assembled by a common V(D)J recombination activity during the precursor (pre-) stages of B and T cell differentiation. Within pre-B and pre-T cell populations, the V(D)J recombination process is controlled in several contexts including lineage-specificity (e.g., complete assembly of Ig variable region genes in B cells but not T cells), developmental stage-specificity (e.g., assembly of Ig heavy chain variable region genes before those of Ig light chains), and allelic exclusion (reviewed by [2–5]). Since there is a common VDJ recombinase, the regulation of Ig or TCR gene segments that are assembled in particular cells and stages within lymphoid lineages must be effected by modulating the accessibility of substrate gene segments to the recombinase (6).

V(D)J recombinational accessibility of both Ig and TCR variable region gene segments has been correlated with a number of factors including their transcriptional activity (7–12). Direct evidence that transcriptional control elements or closely associated sequences can function in *cis* to promote V(D)J recombinational accessibility was provided by a transgenic recombination substrate analyses (9). These studies employed a TCR-β minilocus transgene ($VDJ_\beta C_\mu$ substrate; see Fig. 1) that lacked known transcriptional enhancer elements and that was neither transcribed nor rearranged in lymphoid or non-lymphoid tissues. However, addition of a DNA segment that contained the intronic Ig heavy chain enhancer (E_μ) element ($V_\beta DJ_\beta E_\mu C_\mu$ substrate; see Fig. 1) promoted efficient D to J_β construct rearrangements in most developing lymphocytes.

Various lines of evidence indicate that, for both Ig and TCR loci, V to DJ rearrangements are the most stringently regulated event in the V(D)J recombination process (4, 5, 13, 14). In this context, the transgenic $VDJ_\beta E_\mu C_\mu$ minilocus studies also provided evidence for tissue specific elements that control the V_β to DJ_β rearrangement step. Thus, although this transgene underwent D to J_β rearrangements in most developing B and T cells, construct V_β to DJ_β rearrangements were almost exclusively limited to T lineage cells (9). Therefore, additional elements within this construct appear to differentially control accessibility of the construct V_β gene segment in T vs. B cells. Similar specificity of rearrangement also was observed with an independent TCR-β transgenic recombination substrate (15).

The exact mechanism by which transcriptional control elements function to confer V(D)J recombinational accessibility remains unclear. In particular, a continuing question has been whether transcription per se of V gene segments is sufficient to generate their recombinational accessibility in developing lymphoid cells. The strict enhancer dependence

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and clear lineage-specificity with respect to V_β to DJ_β rearrangements of the TCR-β minilocus recombination substrate provides a model system for in depth studies of the molecular mechanisms of these processes in normal developing lymphocytes. Thus, we have now addressed these issues by assaying for the ability of an unrelated lymphoid-specific enhancer, the minimal TCR-β enhancer (E_β), to promote rearrangement of the $V_\beta DJ_\beta C_\mu$ minilocus and by comparing the transcriptional activity of the V_β and DJ_β portions of the various versions of this substrate in developing B and T lineage cells.

Materials and Methods

Substrate Construction. The recombination substrate $VDJE_\beta C_\mu$ was assembled from genomic DNA fragments encoding TCR-β $V_\beta 14$, $D_\beta 1.1$, $J_\beta 1.1$, and 1.2, TCR-β enhancer, and the C_μ regions as previously described (9, 16) except that the 1.1-kb NaeI–EcoRI IgH enhancer fragment of the $VDJ_\beta E_\mu C_\mu$ construct (9) was replaced with the 560-bp NcoI–HpaI fragment containing the murine TCR-β enhancer (see Fig. 1).

Generation and Analysis of Transgenic Mice. Transgenic mice were generated by injection of the $VDJ_\beta E_\beta C_\mu$ construct into B6/CBA × B6/CBA F1 zygotes as described previously (9). Integration and copy number of transgenic constructs in the various mice was determined by quantifying the level of the 6-kb $V_\beta 14$-hybridizing BglII fragment specific for the transgene by Southern blotting analyses of tail or tissue DNA (see Fig. 2). The transgenic founder mouse no. 5 harbored two independently segregating integrants of the construct, which were propagated as lines no. 5 (60 copies) and 5.1 (5 copies); founder mouse no. 2 was propagated to establish line 2 (25 copies). Founder no. 6 carried one to two copies of the construct in all analyzed tissues, but did not transmit the gene to >50 offspring analyzed. This mouse was killed at 6 mo of age for analysis.

B and T cell purification. B and T cells were purified from transgenic mice by the "panning" procedure (17). B cells were isolated as cells adherent to 10-cm polystyrene bacterial petri dishes (Fisher Scientific Co., Pittsburgh, PA) coated with 250 ng/ml polyclonal goat anti-mouse Ig(G+A+M) (Zymed Laboratories, Inc., S. San Francisco, CA); T cells were isolated as nonadherent cells after two rounds of incubation on plates coated with 10 μg/ml goat anti-mouse Ig(G+A+M). B cells were 95 to 98% pure, as determined by FACS® analysis (FACScan®; Becton Dickinson & Co., Mountain View, CA) using FITC-conjugated rat anti-mouse B220 and PE-conjugated rat anti-mouse CD5 or a combination of PE-conjugated rat anti-mouse CD4 and FITC-conjugated rat anti-mouse CD8 (PharMingen, San Diego, CA) (data not shown). T cells were 80–95% depleted of B cells as determined by FACS® analysis (40–90% $CD5^+$ cells, depending on the non-T, non-B cells in the starting population).

Establishment and Analysis of Lymphoid Bone Marrow Culture (LBMC)[1]. Pre-B cell cultures were established from bone marrow of 3–6-wk-old mice as described previously (18, 19). After 3–4 wk, nonadherent cells were harvested from the cultures for analysis, or 250 U/ml of recombinant human IL-7 was added to the cultures to expand pre-B cells (gift of Stephen Gillis; Immunex Corp., Seattle, WA [20, 21]). After 1 wk in IL-7–supplemented medium, the nonadherent cells were harvested and contaminating adherent stromal cells were removed by incubating the cells on fresh plates for 24 h. Analyzed cells represented pre-B cells based on expression of characteristic surface markers ($BP1^+/CD43^+/B220^+/sIg^-$; [22]) as determined by FACS® analyses after staining with FITC- or PE-conjugated, monoclonal rat anti-mouse BP1, CD43, B220, or IgM (PharMingen), as well as by RAG-1, RAG-2, and $\lambda 5$ expression as determined by Northern blot analyses (23).

A-MuLV-transformed Cell Lines. A-MuLV transformation of adult bone marrow was performed as described previously (24). The cell lines represented the pre-B stage as determined by surface expression of B220 on early passages of the cells, rearrangement of endogenous IgH loci, and expression of RAG-1 and -2 transcripts (data not shown).

Southern Blotting Analysis. Genomic DNA was prepared from tissues and cell lines, digested with BglII and analyzed by Southern blotting procedures as previously described (9, 16). The $VDJ_\beta E_\beta C_\mu$ transgenic line 5 had aberrant integrations resulting in bands that migrate with rearranged products, and could not be clearly analyzed for V(D)J rearrangement by Southern blotting. Therefore, this line was primarily analyzed by PCR methods. Quantitation of Southern blots were performed either on a phosphorimager or densitometer (Molecular Dynamics Inc., Sunnyvale, CA).

PCR Analysis. PCR analysis of the samples were performed as previously described using Amplitaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) (25). Rearrangements in B and T cells were assayed by amplification at: 92°C, 1 min; 55°C, 2 min; 72°C, 3 min for 32 cycles. Rearrangements in A-MuLV–transformed cells and LBMC were examined after amplification at: 92°C, 1 min; 55°C, 2 min; 72°C, 3 min for 40 cycles to detect low frequency V(D)J rearrangements. Oligonucleotide primers to detect rearrangements were: 5′D_β: 5′-GTGAAGCTTCCTTCCTTATCTTCA-ACTC-3′; TCR-β: 5′-GGAATTCTATATCTCTTTGCTGGAATC-3′; 5′C_μ: 5′-TCGGAATTCGGGTATTGGAAAATAATT-3′; $V_\beta 14$: 5′-TCTAAGCTTAAATCAAGCCCTAACCTCTAC-3′. To detect D-J rearrangements, 5′D_β and 5′C_μ (primer set 1DJ) or TCR-β (primer set 2DJ) oligos were used. To detect V(D)J rearrangements, $V_\beta 14$ and 5′C_μ (primer set 1VDJ) or TCR-β (primer set 2VDJ) oligos were used. PCR-amplified products using 5′D_β and 5′C_μ or $V_\beta 14$ and 5′C_μ were analyzed after BamHI digestion, to allow better resolution of the products by gel-electrophoresis. Amplified products were separated on a 1.4% Tris-borate buffered agarose gel, transferred onto zeta-probe membrane (Bio-Rad Laboratories, Inc., Richmond, CA) by alkaline transfer and assayed for hybridization to an oligonucleotide probe to $J_\beta 2$ (5′-AAAGCCTGG-TCCCTGAGCCGA-3′) as previously described (25).

S1 Nuclease Protection Assay. DNA fragments used to generate S1 nuclease protection probes were cloned into pBluescript SK II (Stratagene, La Jolla, CA). ^{32}P-radiolabeled probes were subsequently generated by in vitro transcription using T3 or T7 polymerase (Stratagene), as previously described (26). The $V_\beta 14$ S1 probe was previously described (9). The D-J_β region probe spans the XbaI–EcoRI fragment from the $VDJ_\beta E_\beta C\mu$ construct. The GAPDH probe was made from amplifying position 945-1261 of GAPDH from murine liver cDNA by PCR and cloning the amplified fragment into pBluescript SKII (Stratagene) (Zhang, J., E. Oltz, and F. Alt, unpublished data and reference 27). Approximately 2–50 μg of total RNA was hybridized to the probes, digested with S1 nuclease and resolved on a denaturing acrylamide gel as previously described (9). Yeast tRNA (10 μg) was added to reactions that employed lower amount (2 μg) of cellular RNA.

[1] *Abbreviation used in this paper:* LBMC, lymphoid bone marrow culture.

Results

Generation of VDJβEβCμ Transgenic Mice. We generated the VDJβEβCμ recombination substrate by adding a 560 bp DNA segment that contained the minimal Eβ element to the same site of the VDJβCμ substrate that we previously added the 1126bp Eμ-containing fragment to create the VDJβEμCμ construct (Fig. 1). Three VDJβEβCμ transgenic lines were established that, based on Southern blotting analyses of BglII-digested DNA for hybridization to a Vβ-specific probe, contained ~25 (line 2), 60 (line 5), and 5 (line 5.1) copies of the construct (Table 1 and Fig. 2 *B*). Transgenic founder mouse no. 6 carried a low copy number of the construct in all analyzed tissues (Table 1 and Fig. 2 *B*) but did not transmit the gene. Therefore, this animal was sacrificed at 6 mo of age for analysis. Lines 5, 5.1, and 2 also harbored incomplete integrations of the transgenes that were evident as Vβ-specific BglII fragments of unpredicted sizes (see below) that were unique to each line (Fig. 2; bands denoted with asterisks). The hybridization intensity of these fragments did not change in the various studied tissues (i.e., lymphoid vs. nonlymphoid); therefore they appear to be inactive with respect to recombination and were not further characterized.

The Eβ Element Promotes V(D)J Recombinational Accessibility. DNA from various tissues of the VDJβEβCμ transgenic mice was digested with BglII and analyzed for construct rearrangements by Southern blotting using a probe specific for Vβ14 (Fig. 2 *A*; reference 9). This analysis detects the unrearranged, DJ-rearranged, and VDJ rearranged VDJβEβCμ transgenes as Vβ14-hybridizing BglII fragments of 5.5, 10.5, and 6.5 kb, respectively; whereas the endogenous Vβ gene yields a 3.7 kb hybridizing fragment (Fig. 2 *A*). We detected construct DJ and V(D)J rearrangements at substantial levels in DNA from thymus of all VDJβEβCμ transgenic lines and at somewhat lower, but still significant, levels in DNA from lymph nodes and spleen; a low level of construct DJ rearrangements was also detectable in bone marrow of these lines (Fig. 2 *B*, Table 1, and data not shown). No significant rearrangement of the construct was observed in DNA from nonlymphoid tissues beyond the very low levels attributable to circulating lymphocytes (Fig. 2 *B*; representative data is shown).

The pattern and relative extent of DJ and V(D)J rearrangements of the VDJβEβCμ transgenes was similar to that previously observed for the VDJβEμCμ transgenic lines (9) (representative data for VDJβEμCμ lines 382 and 379 are shown in Fig. 2, *B* and *C*). Significantly, we cannot detect rearrangement of the VDJβCμ construct, which lacks a known enhancer element (Fig. 1), in either lymphoid or nonlymphoid tissues of six independent lines of transgenic mice (reference 9; also, see Fig. 2 *C*; line 1016). Thus physical linkage of a DNA fragment containing the Eβ element, like the Eμ element, targets the associated TCR-β V, D, and J segments for V(D)J recombination.

T Cell-specific V to DJ Rearrangement of the VDJβEβCμ Transgene. We previously observed D to J rearrangements of the VDJβEμCμ transgene in both B and T lineage cells but found construct VDJ rearrangements only in T cells (9). To assay for DJ versus VDJ rearrangement of the VDJβEβCμ substrate in B versus T cells, we used panning procedures to purify splenic and lymph node B and T cells from the four VDJβEβCμ lines and the 382 VDJβEμCμ line; purified B cells were greater than 95% pure as determined by FACS® analysis, while T cells were 80–95% depleted of B cells (data not shown). DNA isolated from purified B and T cells was assayed for rearrangement of the transgenic substrate by a PCR assay that employed primer combinations that specifically detect minilocus DJ and VDJ rearrangements (Fig. 3 *A*). DNA concentration and the efficiency of the PCR reaction was controlled by examining the PCR amplification of a separate DNA fragment in the same reaction (Fig. 3 *A*; legend). In addition, the level of rearrangement in thymic DNA of a given

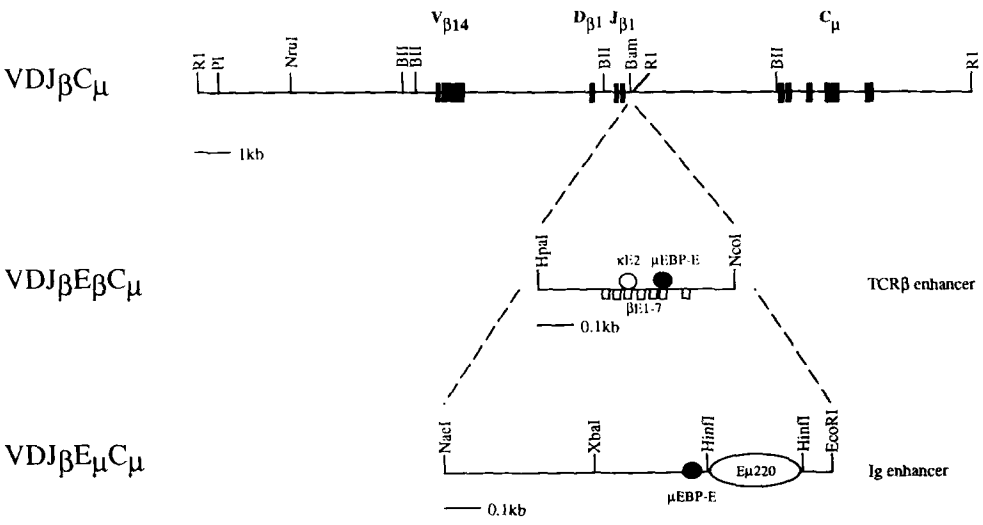

Figure 1. TCR-β minilocus recombination substrates. Partial restriction maps of the TCR-β minilocus recombination substrates that contained either no enhancer (VDJβCμ), a 560 bp DNA fragment that contains the minimal TCR-β enhancer (VDJβEβCμ) or an 1126-bp DNA fragment that contains the Eμ element (VDJβEμCμ). Restriction endonuclease site are indicated as follows: RI, EcoRI; PI, PvuI; BII, BglII; Bam, BamHI. Known DNA motifs important for transcriptional enhancement are shown in the Eβ element (31, 54). The μEBP-E element is also found outside of the core Eμ elements as indicated (30, 31). Note that the inserted elements are drawn on a different scale than the base construct.

Table 1. *Summary of Rearrangement and Transcriptional Characterization of Transgenic Mice Harboring Variations of the TCR-β Recombination Minilocus*

Transgenic lines	Enhancer	Copy number	DJ rearrangement				Percent DJ rearrangement			VDJ rearrangement				Percent VDJ rearrangement	Germline DJ transcripts				Germline Vβ14 transcripts			
			B	T	LBMC	AMuLV	T	LBMC	AMuLV	B	T	LBMC	AMuLV	T	B	T	LBMC	AMuLV	B	T	LBMC	AMuLV
379*‡	IgH	5	+	+						−	+					+				+		
382*‡	IgH	14	+	+	+	+	25	22	28	−	+		−	19	+	+	+	+		+	+	+
390*	IgH	27		+							+											
392*	IgH	4	+	+		+				−	+		−									
1003*	−	6	−	−						−	−											
1006*	−	8	−	−						−	−											
1010*‡	−	2	−	−	−		0	0		−	−	−			−	−	−		−	−	−	
1013*	−	8	−	−						−	−				−	−			−	−		
1016*‡	−	20	−	−		−	0		0	−	−		−		−	−		−	−	−		−
1018*	−	2	−	−						−	−											
2‡	TCR-β	25	+	+	+		19	15		−	+	−		9	+	+	+		+	+	+	
5‡	TCR-β	60	+	+	+		17	14		−	+	−		NA	+	+	+		+	+	+	
6‡	TCR-β	1	+	+			49			−	+			13								
5.1‡	TCR-β	4	+	+	+	+	39	37	17–40	−	+	−	−	24	+	+	+	+	+	+	+	+

"B" denotes B cells; "T" denotes thymus or T cells; "+" indicates detected rearrangement or transcription; "–" indicates lack of rearrangement or transcription; gaps indicate samples not tested; "‡" indicates transgenic lines used in this study; "*" indicates transgenic animals described in a prior study (9). 17–40 denotes the range in the level of rearrangements observed for four independent A-MuLV lines derived from line 5.1. Copy number and percent rearrangement were determined from quantitation of hybridizing signals on Southern blots in Fig. 2 and not shown using Molecular Dynamics phosphorimager and densitometer.

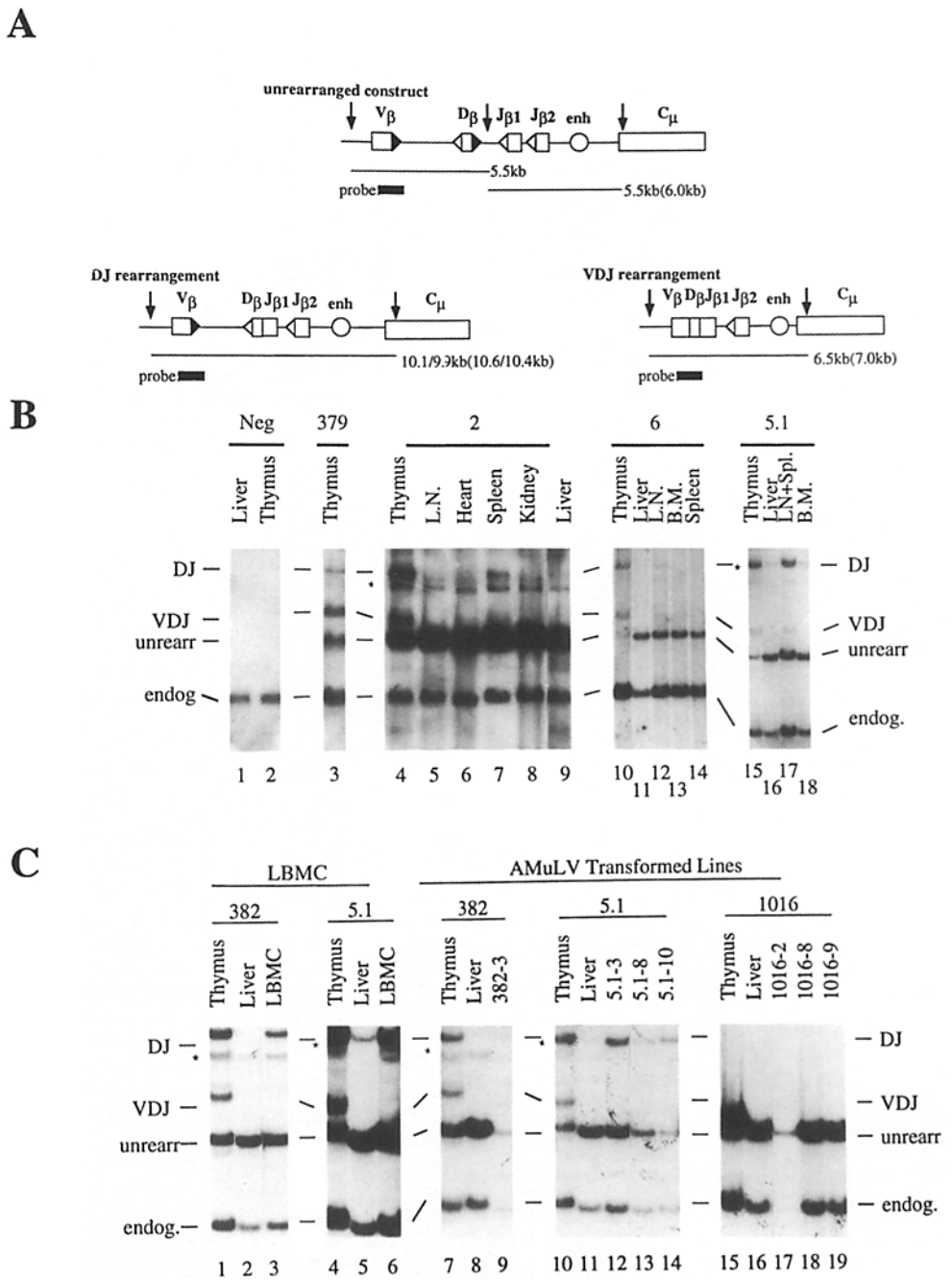

Figure 2. Southern blot analysis of construct rearrangements. (*A*) Schematic of unrearranged DJ or VDJ rearranged miniloci. Arrows represent BglII restriction sites within the miniloci; squares represent variable region gene segment or C_μ region; triangles represent recombination signal sequences. The probe used is indicated and represents the 670-bp AccI fragment spanning the coding and 3′ regions of $V_\beta 14$ (16). The sizes of BglII fragments resulting from V(D)J rearrangements of $VDJ_\beta E_\beta C_\mu$ that hybridize to the probe are denoted; the size of rearrangements of $VDJ_\beta E_\mu C_\mu$ are denoted in parentheses. (*B*) Southern blot analysis of substrate rearrangements in transgenic mice. Genomic DNA (10 μg) isolated from denoted tissues of nontransgenic (lanes *1* and *2*), $VDJ_\beta E_\mu C_\mu$ line 379 (lane *3*), and $VDJ_\beta E_\beta C_\mu$ line 2 (lanes *4–9*), $VDJ_\beta E_\beta C_\mu$ transgenic mouse 6 (lanes *10–14*) and $VDJ_\beta E_\beta C_\mu$ line 5.1 (lanes *15–18*) was digested with BglII and analyzed by Southern blotting for hybridization to the ^{32}P-radiolabeled $V_\beta 14$ probe. Transgene-derived bands that correspond to unrearranged $V_\beta 14$ segments, DJ rearrangements, and VDJ rearrangements, as well as the band that corresponds to the unrearranged endogenous $V_\beta 14$ segment, are indicated. Asterisks indicate bands that derive from aberrant integrations of the construct. LN, lymph node, Spl, spleen, BM, bone marrow. Note that the absolute levels of detected rearrangements in DNA from LN and Spl from some lines is variable, generally due to the proportion of nonlymphoid cells in these tissues. (*C*) Southern blot analysis of DNA from LBMC and A-MuLV-transformed cell lines generated from transgenic mice. DNA isolated from LBMC generated from the $VDJ_\beta E_\mu C_\mu$ transgenic line 382 (lane *3*) and $VDJ_\beta E_\beta C_\mu$ line 5.1 (lane *6*), and A-MuLV transformed cell lines generated from lines 382 (lane *9*), 5.1 (lanes *12–14*) and $VDJ_\beta C_\mu$ line 1016 (lanes *17–19*) are compared with DNA isolated from thymus (lanes *1*, *4*, *7*, *10*, and *15*) and liver (lanes *2*, *5*, *6*, *8*, *11*, and *16*) of respective transgenic lines by Southern blot analysis as described in *B* (See Table 1).

line was used as a standard to estimate the relative level of rearrangements in DNA from purified B and T cells of that line whereas liver DNA from the line was used as a "negative" control. We note that even though such PCR assays are only considered "semiquantitative", we obtained results quite consistent with those obtained by the more quantitative Southern blotting assay. However, the absolute detection level of the PCR assay is at least 10-fold greater.

We detected no DJ or V(D)J construct rearrangements by this sensitive PCR assay in either thymic or splenic DNA of mice transgenic for the $VDJ_\beta C_\mu$ construct (Fig. 3 *B*, line *1010*). In $VDJ_\beta E_\beta C_\mu$ and $VDJ_\beta E_\mu C_\mu$ lines, we detected levels

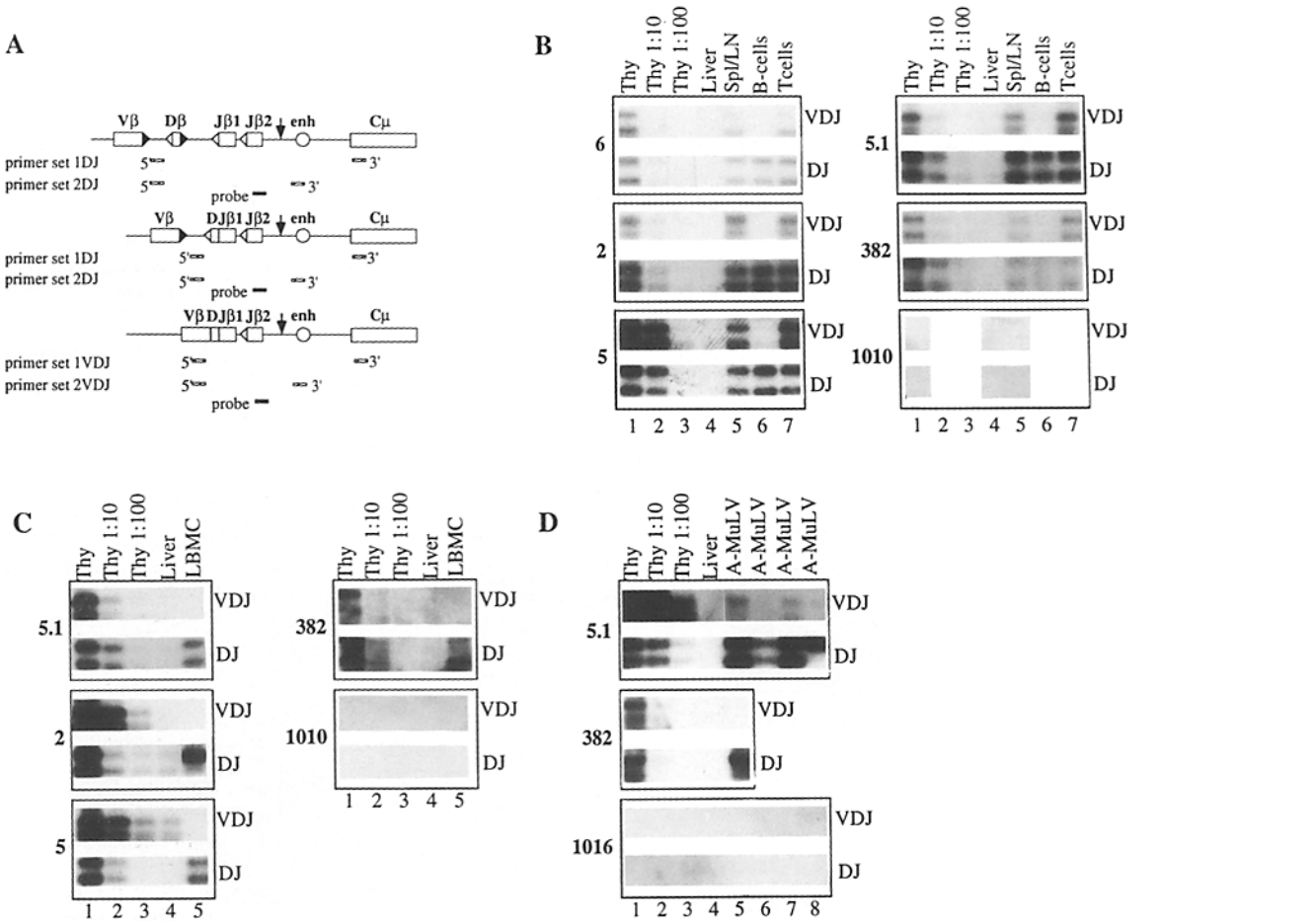

Figure 3. PCR analysis of construct rearrangements. (*A*). Schematic for primers used to detect substrate rearrangements. Primer set 1DJ (*top* and *middle*) and 1VDJ (*bottom*) and primer set 2DJ (*top* and *middle*) and 2VDJ (*bottom*) were designed to specifically amplify recombination substrate sequences. Diagrams depict rearrangement to $J_\beta 1.1$. Arrows depict BamHI sites in the substrate and amplified products. Primer set 1 was used to detect rearrangements of transgenic recombination substrates $VDJ_\beta E_\mu C_\mu$ (line 382) or $VDJ_\beta C_\mu$ (lines 1010 and 1016). Amplified products from PCR of DJ and V(D)J rearrangements using primer set 1 were digested with BamHI before analysis. Primer set 2 was used to detect rearrangements from genomic DNA with transgenic substrate $VDJ_\beta E_\beta C_\mu$ (mouse 6, lines 2, 5, and 5.1). Amplified products obtained with either set of primers were analyzed by Southern blot for hybridization to an oligonucleotide probe to $J_\beta 1.2$. Predicted BamHI-digested amplified products of D_β to $J_\beta 1.1$ and $J_\beta 1.2$ rearrangements using primer set 1DJ (*top* and *middle* diagrams) result in hybridizing bands of 450 and 250 bps, respectively; a 1.1-kb BamHI-digested PCR product of an unrearranged substrate also is detected. BamHI digested PCR products of substrate V(D)J rearrangements using primer set 1VDJ result in products of 470 and 330 bps (*bottom*). PCR using primer set 2DJ of $VDJ_\beta E_\beta C_\mu$ substrate result in amplified products of 520 and 380 base pairs for $DJ_\beta 1.1$ and $DJ_\beta 1.2$ rearrangements, respectively, and 1.2 kb for unrearranged substrate (*top* and *middle*). Amplification of $VDJ_\beta E_\beta C_\mu$ V(D)J rearrangements using primer set 2VDJ result in products of 470 and 330 bps (*bottom*). (*B*) Rearrangement of recombination substrates in purified B and T cells of transgenic mice. 0.5 μg of genomic DNA from transgenic mouse no. 6, and lines 2, 5, 5.1, 382, and 1010 were analyzed by PCR for DJ and VDJ rearrangement of the transgenic recombination substrate as described above. PCR products of DJ and V(D)J rearrangements are shown. PCR to detect DJ and VDJ rearrangements for all samples of each line were performed in parallel, using the same DNA sample in appropriate reactions. The level of relative rearrangement in the samples should be compared to thymus for each line. Thymus DNA for each transgenic line was diluted into liver DNA and amplified in parallel with DNA from B and T cells, since different copy numbers of the recombination substrate affects the sensitivity of the PCR assay for each transgenic line. Amplification of unrearranged substrate using primer set 1DJ and set 2DJ or coamplification of a segment of an unrelated gene for lines 2, 5.1, and 1010 (precursor lymphocyte–specific regulatory light chain [PLRLC] [55]; data not shown) were used to demonstrate equal efficiency within each set of amplification reactions. (*C*) Rearrangement of recombination substrates in LBMC generated from transgenic mice. PCR were performed on genomic DNA from denoted sources as described in *B*. Reactions of each set were determined to be equally efficient by amplifying genomic sequence from PLRLC simultaneously (data not shown). (*D*) Rearrangement of recombination substrates in A-MuLV–transformed pre-B cell lines generated from transgenic mice. PCRs were performed on 0.5 μg of genomic DNA from denoted sources as described in *B*. PLRLC sequences were amplified simultaneously, showing that samples in each set of reactions were amplified with equal efficiency (data not shown).

of construct DJ rearrangements in DNA from purified peripheral B cells (Fig. 3 *B*, lane *6*) and T cells (Fig. 3 *B*, lane 7) that were usually comparable in magnitude to the levels found in the corresponding thymic DNA (Fig. 3 *B*, lanes *1–3*). However, in all tested lines, V to DJ rearrangement of the $VDJ_\beta E_\beta C_\mu$ and $VDJ_\beta E_\mu C_\mu$ transgene were found at substantial levels in peripheral T cells (Fig. 3 *B*, lanes 7), but were nearly undetectable in DNA from peripheral B cells (Fig.

3 *B*, lanes *6*). We observed only a very low level (more than 100× less than that of the corresponding thymus) of D-J and V(D)J rearrangements in liver DNA of mice transgenic for $VDJ_\beta E_\beta C_\mu$ and $VDJ_\beta E_\mu C_\mu$ (Fig. 3, *B* and *C*, lane *4*) that likely result from circulating lymphocytes. Therefore, we conclude that the 560-bp minimal E_β element targets rearrangement of this recombination substrate with a tissue-specific profile similar to that observed in the context of the $E\mu$ element.

To confirm that the lack of construct V(D)J rearrangements observed in peripheral B cells was also a characteristic of pre-B cells, we assayed for construct rearrangements in LBMC pre-B cells and in A-MuLV transformed cell lines established from the various transgenic mouse lines. Long-term LBMC were established from $VDJ_\beta E_\beta C_\mu$ transgenic lines 5, 5.1, and 2, $VDJ_\beta E_\mu C_\mu$ line 382, and $VDJ_\beta C_\mu$ line 1010. The LBMC were propagated in media supplemented with recombinant human IL-7 (19–21) for 1 wk to expand immature pre-B cells. Nonadherent cells harvested from the LBMC were determined to represent pre-B cells based on expression of various surface markers and of RAG-1 and -2 transcripts (data not shown and reference 23). A-MuLV transformed B cell lines were established from bone marrow of $VDJ_\beta E_\beta C_\mu$ transgenic line 5.1, $VDJ_\beta E_\mu C_\mu$ line 382, and $VDJ_\beta C_\mu$ line 1016 (24). These cell lines also were determined to represent pre-B cells based on expression of characteristic surface markers, rearrangement of endogenous IgH loci, and expression of RAG-1 and -2 transcripts (data not shown).

We readily detected DJ, but not V(D)J construct rearrangements by Southern blotting analyses of DNA from LBMC and A-MuLV transformed cell lines established from the $VDJ_\beta E_\beta C_\mu$ and $VDJ_\beta E_\mu C_\mu$ transgenic lines (Fig. 2, *C* lanes *3, 6, 9, 12, 13*, and *14*; and Table 1). In contrast, neither DJ nor VDJ rearrangements were detected by a similar analysis of DNA from $VDJ_\beta C_\mu$ transgenic A-MuLV transformants (Fig. 2 *C*, lanes *17–19*). We also assayed for construct DJ and V(D)J rearrangements in DNA from the various LBMC and A-MuLV pre-B cell lines by the more sensitive PCR method to detect potential construct rearrangements that occurred below the detection level of the Southern blotting assay. Thymus and liver DNA samples from the corresponding lines again were used as controls. Even with this sensitive assay, we could not detect rearrangements of the $VDJ_\beta C_\mu$ substrate in DNA from LBMC and A-MuLV transformed cell lines generated from $VDJ_\beta C_\mu$ transgenic mice (Fig. 3 *C*, line 1010; Fig. 3 *D*, line 1016). In contrast, substantial levels of DJ substrate rearrangement, comparable to that of the corresponding thymic DNA, were detected in DNA from all LBMC and A-MuLV–transformed cell lines established from $VDJ_\beta E_\beta C_\mu$ and $VDJ_\beta E_\mu C_\mu$ transgenic mice (Fig. 3 *C*). However, we again could not detect significant levels (levels were at least 100-fold less than that of the corresponding thymus sample) of construct VDJ rearrangements in LBMC and only extremely low levels in some A-MuLV transformants from $VDJ_\beta E_\beta C_\mu$ and $VDJ_\beta E_\mu C_\mu$ transgenic mice (Fig. 3, *C* and *D*).

Enhancer Dependent Transcription of the Unrearranged Construct D-Jβ Region in B Lineage Cells. To quantify expression of transcripts from the D-J_β portion of the transgenic recombination substrates, we employed an S1 nuclease protection assay that used a probe which included the D-J_β and E_β regions of the $VDJ_\beta E_\beta C_\mu$ construct (Fig. 4 *A*, *top*). A combination of S1 nuclease protection and primer extension analyses of thymic RNA from $VDJ_\beta E_\mu C_\mu$ transgenic mice indicated that transcripts initiate from multiple sites in the region of the construct between the D and $J_\beta 1$ segment (Ferrier, P., and F. Alt, unpublished data). Thus, hybridization of transcripts that span the entire region of the probe will result in a 950-bp protected fragment in RNA from $VDJ_\beta E_\beta C_\mu$ transgenic lines and a 490-bp fragment in RNA derived from $VDJ_\beta E_\mu C_\mu$ or $VDJ_\beta C_\mu$ lines, whereas transcripts that initiate internal to the probe protect a series of smaller fragments. Potential transcripts from $VDJ_\beta E_\beta C_\mu$ $DJ_\beta 1$ or $DJ_\beta 2$ rearrangements would protect probe fragments of 870 or 740 bp whereas those from $VDJ_\beta E_\mu C_\mu$ or $VDJ_\beta C_\mu$ rearrangements would protect fragments of 310 or 180 bp. As a control, we also quantitated expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts simultaneously or in parallel (Fig. 4 *B*).

We did not detect transcripts of the unrearranged construct D-J region in RNA from thymus, liver, LBMC, or A-MuLV transformants derived from $VDJ_\beta C_\mu$ transgenic animals (reference 9 and Fig. 4 *B*, *top*; lanes *5–9*). In contrast, we detected D-J region construct transcripts in RNA from thymus, LBMC and A-MuLV transformants, but not liver, from both $VDJ_\beta E_\mu C_\mu$ and $VDJ_\beta E_\beta C_\mu$ transgenic mice (Fig. 4 *B*, *top*; lanes *2–4*, *10–15*, and *18–24*). Thus, DNA fragments containing either the E_μ or E_β enhancers activate transcription through the D_β and J_β portion of the transgenic minilocus in both pre-B cells and thymocytes.

Enhancer-dependent Transcription of Unrearranged Substrate Vβ Segments in B Lineage Cells. We employed a probe spanning the 3′ end of the construct $V_\beta 14$ segment (Fig. 4 *B*, *bottom*) in S1 nuclease protection assays to detect transcripts derived from this portion of the transgenic constructs; this probe detects transcripts from unrearranged and rearranged $V_\beta 14$ segments as protected fragments of 340 and 90 bp (Fig. 4 *A*, *bottom*). Because this probe does not distinguish between construct-derived and endogenous V_β-containing transcripts; transgene expression is detected by quantitative comparison of transcript levels in corresponding RNA samples from transgenic and nontransgenic sources (9).

We detected transcripts of germline and rearranged $V_\beta 14$ segments at levels well above nontransgenic background in RNA from thymus, but not liver, of all mice transgenic for $VDJ_\beta E_\mu C_\mu$ or $VDJ_\beta E_\beta C_\mu$ constructs (Fig. 4 *B*, *bottom*, lanes *2–4*, *10–15*, and *18–24*). In contrast, we did not detect levels of $V_\beta 14$ transcripts in thymus RNA of $VDJ_\beta C_\mu$ transgenic mice above those of nontransgenic mice (reference 9, Fig. 4 *B*, *bottom*, lanes *5–9* and data not shown). Thus, the E_β enhancer, like the E_μ enhancer (9), can activate transcription of the unrearranged construct V_β segment in thymocytes. Surprisingly, purified peripheral B cells from $VDJ_\beta E_\mu C_\mu$ or $VDJ_\beta E_\beta C_\mu$ mice expressed levels of $V_\beta 14$ transcripts that were reproducibly as high or higher than those found in RNA from the corresponding thymuses (Fig. 4 *C*). To determine

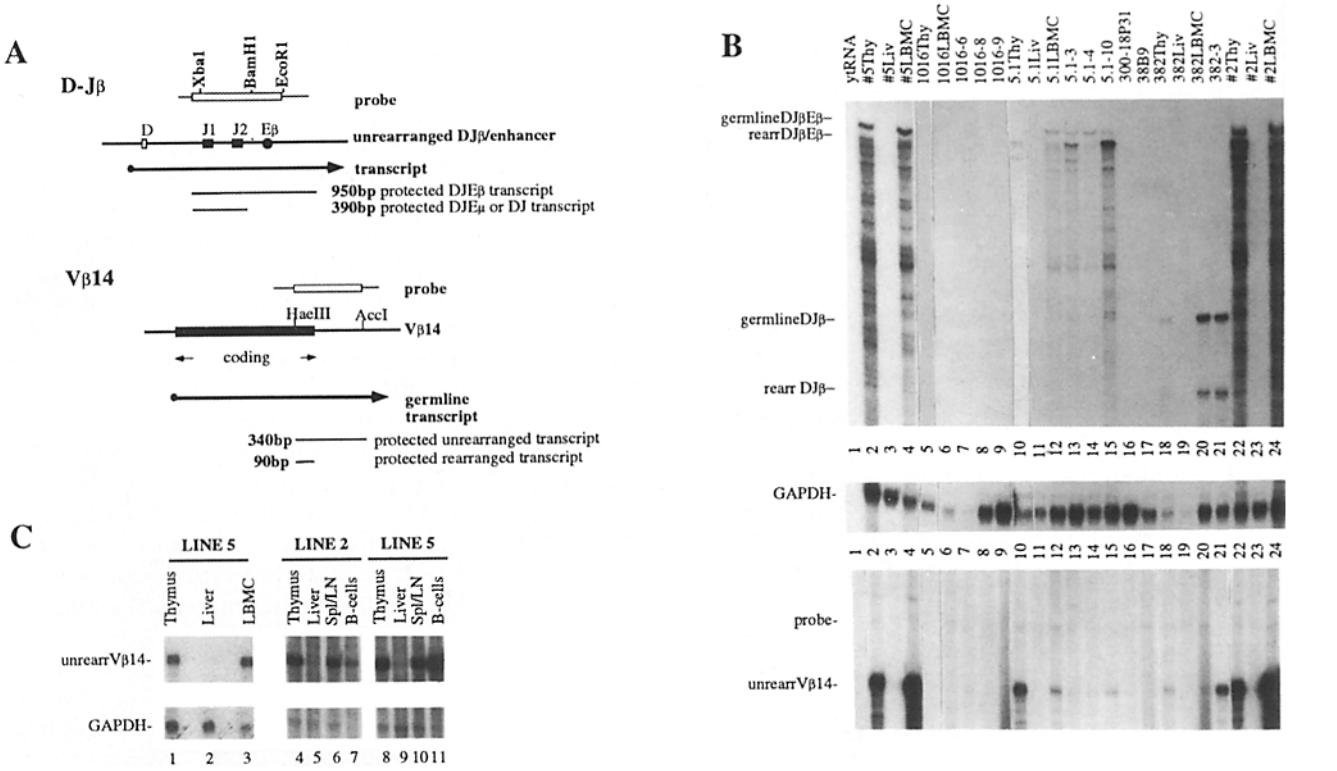

Figure 4. Expression of the transgenic recombination substrates. (*A*) (*Top*): Schematic of S1 probes used to detect transcripts from the minilocus D-J regions. Other details concerning construct-specific transcripts are in the results section. Although transcripts of endogenous DJ_β or VDJ_β rearrangements would protect 310 and 180 bp fragments of this probe, we could not unequivocally identify the presence of these transcripts except in normal thymic RNA (data not shown). (*Bottom*): the probe to detect $V_\beta14$ transcripts; sizes of protected fragments resulting from transcripts of rearranged or unrearranged sequences are indicated. Other details are in the methods or results section. (*B*) Transcripts of the D-J (*top*) and $V_\beta14$ (*bottom*) regions of the recombination substrate in tissues and cell lines generated from transgenic mice. 10 μg of total RNA from indicated sources were simultaneously assayed for hybridization to the substrate DJ region (*top*) and GAPDH (*middle*) probes; hybridization to the $V_\beta14$ probe (*bottom*) was assayed in with duplicate RNA samples. Bands corresponding to unrearranged and rearranged D-J transcripts and unrearranged $V_\beta14$ segments are indicated. Bands corresponding to undigested probe in $V_\beta14$ protection assays are denoted. (*C*) Transcripts of the $V_\beta14$ region of the recombination substrate in long-term LBMC and purified B cells from transgenic mice. Approximately 2 μg of total RNA was assayed from thymus, liver, non-adherent cells from long-term LBMC and purified B cells were assayed for transcription of the unrearranged $V_\beta14$ segment. Transcription of GAPDH was assayed simultaneously.

whether the V_β portion of these transgenes was also expressed in pre-B cells, we assayed RNA from LBMC. Again, we reproducibly detected unrearranged $V_\beta14$ gene transcripts in LBMC (cultured with or without added IL-7) established from all tested $VDJ_\beta E_\mu C_\mu$ or $VDJ_\beta E_\beta C_\mu$ lines at levels that often approached those of the corresponding thymic RNA; these transcripts were enhancer dependent as none were detected in LBMC established from $VDJ_\beta C_\mu$ transgenic mice (with IL-7, Fig. 4 *B bottom*, lanes *6, 12, 20*, and *24*; without IL-7, Fig. 4 *C*, lane *3*). Because the V_β expression levels in cultured pre-B, peripheral B, and thymic T cells from a given transgenic line were roughly proportional to $VDJ_\beta E_\mu C_\mu$ and $VDJ_\beta E_\beta C_\mu$ copy numbers (which range from a few to about 100), it also would appear that most copies of the transgene are expressed in each of these cell types (see Fig. 4, *B* and *C*). As with $VDJ_\beta E_\mu C_\mu$ mice (9), we could not readily detect $V\beta$ transcripts in total bone marrow of the $VDJ_\beta E_\beta C_\mu$ mice (data not shown). Assuming that normal pre-B cells express this transcript at the same level as those in LBMC, the difficulty in detecting these transcripts in total bone marrow is consistent with the low level of B lineage cells in this tissue (28).

Analyses of $V_\beta14$ expression in A-MuLV transformants derived from the various transgenic lines yielded results that were generally in accord with those obtained from normal B lineage cells. Thus, we also detected unrearranged $V_\beta14$ transcripts in $VDJ_\beta E_\mu C_\mu$ and $VDJ_\beta E_\beta C_\mu$ A-MuLV transformants (see Fig. 4 *B*). However, the expression level of these transcripts often was lower than that of the corresponding LBMC culture (see Fig. 4 *B*), possibly due to downregulation of transgene expression in these transformed lines. Downregulation of the expression of some endogenous lymphoid-specific genes has been clearly documented in such lines (29). We did not detect unrearranged $V_\beta14$ transcripts in RNA from nontransgenic A-MuLV transformants (see Fig. 4 *B*, lanes *16* and *17*). We also did not detect these transcripts in 2 of 3 tested A-MuLV transformants from the $VDJ_\beta C_\mu$ transgenic line; in the other line they occurred at extremely low levels (see Fig. 4 *B*, lanes *7–9*).

We did not detect protected fragments that clearly cor-

responded to transcripts of rearranged V_β segments in RNA from LBMC or peripheral B cells. However, we occasionally observed protected fragments that migrated roughly at this size (80 bp) in some LBMC sample (data not shown). The latter fragments were likely artifactual due to probe or RNA degradation because they usually were not found in repeat experiments with samples from the same source and because there were no detectable V_β rearrangements in the DNA from these sources. On the other hand, we did detect very low but reproducible levels of protected fragments that corresponded to rearranged V_β transcripts in RNA from A-MuLV transformants derived from $VDJ_\beta E_\beta C_\mu$ mice; these probably derived from the low level of construct V_β rearrangements found in these cell lines (Fig. 3 *C*).

Discussion

The Minimal TCR-β Enhancer Activates D to J TCR-β Minilocus Rearrangements in B and T Lineage Cells. We have demonstrated that addition of the minimal TCR-β enhancer region to the $VDJ_\beta C_\mu$ construct activates its transcription and rearrangement in developing B and T lineage cells in a fashion essentially identical to that which we previously observed for a larger DNA fragment that contains the $E\mu$ element. The "core" E_μ and E_β elements are unrelated; although the E_μ-containing sequence employed in our earlier studies contained one site, μEBP-E (30–32) also found in the core E_β element.

The ability of both the E_μ and E_β enhancer elements to direct rearrangement of the TCR-β minilocus in a *cis*-dominant fashion suggests that these and other Ig or TCR transcriptional enhancers may function as "recombination" enhancers in their endogenous setting. Accordingly, recent gene-targeted mutation studies demonstrated that deletion or replacement of the endogenous E_μ element significantly reduced the rearrangement level of the associated J_H segments (33, 34). However, the E_μ deletion did not eliminate J_H rearrangements. Therefore, it is likely that the $VDJ_\beta C_\mu$ construct lacks additional elements present in the endogenous IgH locus that promote rearrangement independently from the E_μ element but that may act synergistically with E_μ to confer full V(D)J recombinational accessibility. Thus, the $VDJ_\beta C_\mu$ minilocus should provide a system to identify and further characterize such elements.

Enhancer-dependent Transcriptional Activation of the Minilocus Recombination Substrate. The unrearranged $V_\beta 14$ segment and the D-J_β portion of the $VDJ_\beta E_\mu C_\mu$ and $VDJ_\beta E_\beta C_\mu$ but not $VDJ_\beta C_\mu$ transgenes are transcribed in both T and B lineage cells. Thus, the generation of both types of transcripts is enhancer-dependent. Correspondingly, the E_β and E_μ elements can activate multiple promoters over long distances in B and T lineage cells (31, 35–39). Notably, the endogenous TCR D-J_β locus and $V_\beta 14$ gene segment generally are transcribed in T but not B lineage cells. Therefore, transcription of these regions of the TCR-β locus are differentially regulated in the minilocus vs. the endogenous locus, even in the context of the E_β element. As the $V_\beta 14$ segment is further from the E_β element in the transgene than in the endogenous locus, it is unlikely that distance effects with respect to these sequences are responsible for transcriptional activation in B cells. Thus, as previously proposed (31, 36), endogenous elements in addition to the core E_β element likely contribute to T cell–specific transcription of V_β and D-J_β sequences.

Potential Relationships between Transcriptional Activation and V(D)J Recombinational Accessibility. Transcription has been shown to promote homologous recombination (40–42). Likewise, V(D)J recombinational accessibility of endogenous and stably transfected variable region gene segments has been correlated both with transcription (6, 7, 12, 43, 44) and with the *cis*-acting functions of transcriptional enhancer elements (9, 25, 45; this study). However, with respect to V(D)J recombination, the nature of the relationship between these processes has remained unclear. The recombinational enhancement of DJ_β rearrangements conferred by the two different enhancer elements in our substrates again correlates well with their transcriptional enhancement of the same region of the substrate. However, our data suggest a surprising dichotomy between $V_\beta 14$ transcription and rearrangement in B lineage cells.

We detected essentially no rearrangements of the $V_\beta 14$ segments of $VDJ_\beta E_\beta C_\mu$ or $VDJ_\beta E_\mu C_\mu$ transgenes in any normal B lineage cells. Yet, we clearly detected transcription of the unrearranged substrate $V_\beta 14$ segment in both early and late-stage B lineage cells. These results indicate that enhancer dependent transcription of the minilocus Vβ14 segment occurs throughout all stages of early B cell development, including stages that undergo V(D)J recombination of endogenous Ig loci. Several lines of evidence indicate that the transcripts that we have detected originate from the complete transgene substrate, rather than an aberrantly integrated copy. Thus, the transcripts are lymphocyte-specific (indicating enhancer dependence) and correspond in steady-state level in the various lines in rough proportion to the transgene copy number of the line.

Thus, our current studies make the unexpected and novel observation that a transcribed variable region gene segment still may not be accessible to V(D)J recombinase, indicating that, at least in some contexts, transcription per se of a variable region gene segment is not sufficient to promote its recombinational accessibility. A similar situation apparently exists with respect to control of Ig heavy chain class switch recombination; gene targeted mutations that generate constitutive transcription through the Ig Cϵ switch recombination sequences do not promote significant levels of switching to that region in appropriately activated B cells (46).

The potential relationship between transcriptional activation and recombinational accessibility has also been the subject of studies that have taken the converse approach of assaying for rearrangement of apparently nontranscribed substrates. Transiently transfected recombination substrates were shown to recombine in the absence of transcriptional control elements and steady-state transcripts (47). However, such observations may not be directly relevant to normal control mechanisms, as genes on small extra chromosomal substrates are likely subject to different regulatory constraints

than chromosomally integrated genes. In another study, transgenic V_κ and J_κ segments were found to rearrange in the absence of detectable steady-state transcripts from the recombination substrates (48); but levels of detected rearrangements were very low, the absence of transcription was not directly demonstrated, and assays were done only in mature B lineage cells. Therefore, there still is no compelling evidence that a chromosomal locus can undergo efficient V(D)J recombination in the absence of transcriptional activation and/or transcriptional control elements.

Several studies have shown that accessibility is strongly correlated with hypomethylation of substrate loci (49–51). Due to the lack of informative restriction endonuclease sites, we could not directly address this issue with respect to the V_β gene segment portion of our constructs. However, as transcribed genes are usually hypomethylated (52), we would expect this to be the case for the V_β and DJ_β regions of our construct in both B and T lineage cells. Even so, recent targeted mutational analyses of the E_μ element and flanking sequences indicated that hypomethlyation also may not be sufficient to render the endogenous J_H locus fully accessible for V(D)J recombination (34).

In summary, it appears that, at least in some contexts, neither transcriptional activation nor hypomethylation of chromosomal variable region gene segments is sufficient to render them fully accessible for V(D)J recombination. Conversely, efficient rearrangement of nontranscribed or methylated chromosomal variable region gene segments has not been clearly documented. Thus, it remains possible, and even likely, that transcription and/or transcriptional enhancing element plus demethylation may be necessary companions of full V(D)J recombinational accessibility; but that additional factors further modulate the control of this process in some contexts.

Differential Control of D to J and V to DJ Rearrangements. Various lines of evidence indicate that, for both Ig and TCR loci, V to DJ rearrangement is the most strictly regulated V(D)J recombination event, both in the context of tissue specificity and allelic exclusion (12–14, 53). Unlike many other test systems, this aspect of normal control is clearly reproduced in our transgenic recombination substrates, possibly due to the composition of the construct and/or its relatively large size (which may obviate position effects). In this regard, our current findings are consistent with a model in which D and J recombination events are promoted by E_μ functions identical to or closely associated with its transcriptional enhancing activities whereas the more stringently regulated V to DJ joining event may be influenced by additional positive or negative control elements that ensure appropriate specificity.

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