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The V(D)J Recombinational and Transcriptional Activities of the Immunoglobulin Heavy-Chain Intronic Enhancer Can Be Mediated through Distinct Protein-Binding Sites in a Transgenic Substrate

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Immunoglobulin and T-cell receptor gene transcriptional enhancers encompass sequences which stimulate V(D)J recombination of associated variable gene segments. To address the question of whether enhancer-mediated transcriptional activation and recombinational activation depend on the same *cis*-regulatory sequences, we have produced transgenic mice by using recombination substrates containing various mutations in the immunoglobulin heavy-chain intronic enhancer (Eμ). Analysis of substrate rearrangements indicated that specific compound elements including E-box transcriptional motifs are crucial for the recombinational activity of Eμ in the developing B and T lymphocytes. In most cases, a faithful correlation between the levels of substrate germ line transcription and recombination was observed. However, some of the Eμ mutants which were able to activate transcription of the unrearranged substrate were inefficient in stimulating transgene recombination, implying that the latter function depends on molecular events other than the mere activation of transcription and that both activities can be mediated through distinct regulatory sequences. Together, these results support a model in which lymphoid gene enhancers, in addition to providing docking sites for factors that dictate transcriptional accessibility, must have some specific function(s) for activating V(D)J recombination.

Immunoglobulin (Ig) and T-cell receptor (TCR) variable region genes are assembled from homologous variable (V), diversity (D), and joining (J) germ line segments during the early stages of lymphoid B- and T-cell differentiation. This process, known as V(D)J recombination, is regulated at several levels, including the cell lineage as well as the stage of cell differentiation. Thus, Ig genes are completely assembled only in B cells and TCR genes are assembled only in T cells, with Ig heavy-chain (IgH) and TCRδ/γ/β genes being assembled prior to Ig light-chain (IgLκ or IgLλ) and TCRα genes, respectively. Since a unique recombinational activity [referred to as V(D)J recombinase] appears to be expressed in both differentiating B and T cells, the cell type and cell stage specificities of the V(D)J recombination process are currently interpreted in the context of a model which proposes regulated changes in chromosomal accessibility to the recombinase (reviewed in references 1 and 52).

The molecular events which mediate recombinational accessibility are still controversial. Transcription of unrearranged germ line gene segments and/or partially rearranged gene regions commonly correlates with their activation for recombination (for examples, see references 21, 53, 55, 57, and 63). Moreover, the frequency at which introduced gene segments are assembled in pre-B-cell lines appears to increase with flanking gene expression (5, 64). These data led to the hypothesis that transcription of Ig and TCR genes is a prerequisite to their rearrangement. Conceivably, transcription may directly confer DNA recombinational accessibility by inducing critical changes in the local chromatin structure. Alternatively, other mechanisms, including targeted DNA demethylation, may play a key role in determining locus accessibility, thus causing indirectly incidental transcription (28).

Recently, transgenic mouse experiments have stressed the role of Ig and TCR gene transcriptional enhancers on the *cis* regulation of V(D)J recombination (reviewed in reference 61). Thus, DNA fragments containing the IgH, TCRβ, or TCRα gene enhancer not only activate V(D)J recombination within a transgenic recombination substrate but also confer cell-type- and/or cell-stage-specific patterns of rearrangement to the associated germ line segments (9, 18). Moreover, targeted deletion and/or replacement of IgH and IgLκ gene enhancers in mouse embryonic stem cells result in an inhibition of *cis* rearrangements in the resultant B-lineage progeny (11, 56, 60). Ultimately, the definition of specific sequences within lymphoid gene enhancers which stimulate V(D)J recombination may contribute to the understanding of the molecular mechanism(s) involved in recombinational accessibility.

The mouse IgH gene enhancer (Eμ), located in the intron between the J_H and constant Cμ exons, is one of the best-studied transcriptional regulatory elements in higher eukaryotes. Most of the enhancer activity has been localized to a 700-bp *Xba*I-*Eco*RI fragment (Eμ700) that functions in all stages of the B-cell lineage, including early development, and also in T-lineage cells (see references 39 and 58 and references therein). Extensive analysis has identified a complex set of overlapping regulatory elements within Eμ700. Mutation of individual elements or deletion of small regions within Eμ700 generally leads only to a minor decrease in the transcriptional enhancement due presumably to the functional redundancy between the distinct elements. The various elements of Eμ have been shown to be the targets of DNA-binding proteins which act as transcription factors. Notably, molecular cloning of cDNAs encoding factors that bind to the μE1 to μE5 E-box motifs has identified a series of ubiquitously expressed proteins which belong to the helix-loop-helix family of transcription

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factors (reviewed in reference 30). Mutational studies have suggested that the E-box motifs and specific binding factors contribute to the transcriptional activity of Eμ in lymphoid cells (3, 33, 36) while silencing this activity in nonlymphoid tissues (23, 51, 62). Additional elements important for lymphoid cell and/or pre-B-cell specificity include the octanucleotide motif which binds members of the POU domain family of transcription factors (reviewed in reference 50) and the π/μA and μB motifs which interact with members of the Ets protein family (40, 44, 48).

To address the question of whether transcriptional activation and recombinational activation are mediated by the same *cis*-regulatory sequences, we have produced transgenic mice by using recombination substrates containing various mutations in the Eμ enhancer. We have analyzed V(D)J rearrangements, germ line transcription, and levels of DNA methylation of the recombination substrates in lymphoid and nonlymphoid tissues of the transgenic animals. The results have potential implications in respect to the role of lymphoid cell-specific enhancers in inducing V(D)J recombination.

MATERIALS AND METHODS

Construction of the recombination substrates. The wild-type (Eμ700 WT, Eμ460 WT, and Eμ300 WT) enhancer fragments were isolated from plasmid p17.7-14 (17) and correspond to the *Xba*I-*Eco*RI (nucleotides 2877 to 3559 in locus MUSIGCDO7/GenBank, accession number J00440), *Xba*I-*Alu*I (nucleotides 2877 to 3330), and *Pvu*II-*Eco*RI (nucleotides 3258 to 3559) fragments, respectively. The Eμ700 $E3^-E4^-$ mutant (36) was isolated from a plasmid kindly provided by M. Lenardo and D. Baltimore; the Eμ Es^-O^- mutant, corresponding to fragment 19 in reference 33, was isolated from a plasmid kindly provided by T. Kadesch. Construction of the recombination substrates by using blunt-ended Eμ fragments was done as described previously (9); all Eμ fragments were oriented as in reference 18. The nucleotide sequence of each Eμ fragment in the recombination substrates was verified before microinjection.

Probes. The Jβ1 and 3′Dβ probes have been already described (17, 18). The μ800 probe corresponds to an *Eco*RI-*Hin*cI restriction fragment from the Ig J_H-Cμ intron (nucleotides 3559 to 4381 in locus MUSIGCDO7/GenBank). The β-actin probe was produced by PCR amplification using β-actin-specific oligonucleotide primers.

Production of transgenic mice. Linearization, purification, microinjection of the various insert DNAs into fertilized (C57BL/6 × CBA/J)F_2 eggs, identification of the transgenic founders, and production of lines of transgenic mice were done as described previously (18). The characteristics of the transgenic animals in the Eμ1100 WT:14 line have been reported previously (line 382 in reference 18).

Southern blot analyses. Preparation of genomic DNA, restriction enzyme digests, agarose gel electrophoresis, DNA blotting procedures, preparation and ^{32}P labeling of DNA fragments used as probes, and hybridization procedures were done as described previously (18). Quantifications of band intensities were derived from densitometric analyses of autoradiograms, using a model GS300 scanning densitometer (Hoefer Scientific Instruments), and/or hybridized filters, using a Baf 1000 imaging plate device (Fuji). Percentages of total substrate, DJ, and VDJ substrate rearrangements were determined (from Jβ1-hybridized blots) as described previously (9).

Cell separation. Peripheral B and T cells were purified from a mixed cell suspension from transgenic spleens and lymph nodes. B cells were purified by depleting T cells with an anti-Thy1 monoclonal antibody (MAb) plus rabbit complement (Cedarlane, Hornby, Ontario, Canada); T cells were depleted of B cells by using an anti-CD45 (B220) MAb plus Dynabeads (Dynal AS, Oslo, Norway). Cellular purity was monitored by immunofluorescence (fluorescence-activated cell sorting [FACS]) analysis using anti-CD3 and $F(ab')_2$ anti-surface Ig (sIg) fluorescent MAbs.

Mature ($B220^+$ sIg^+) and immature ($B220^+$ sIg^-) B cells were purified by cell sorting using a FACStar Plus (Becton Dickinson). Bone marrow cells were purified from a pool of three adult mice by using a phycoerythrin-conjugated anti-B220 MAb (RA3-6B2; PharMingen) and fluorescein isothiocyanate-conjugated anti-mouse IgG (H+L) $F(ab')_2$ fragment (Jackson Laboratories) which recognizes mouse IgG heavy chains and all mouse light chains. Sorted populations were reanalyzed for purity after sorting and were >98% pure.

RNA PCR assays. RNA PCR assays were done as described previously (9) except that the cDNA was amplified for 24 to 28 cycles (94°C for 1 min, 57°C for 0.5 min, 72°C for 1.5 min). RNAs were treated with DNase I (Promega, Madison, Wis.) prior to reverse transcription. Comparative analyses were performed on cDNAs synthesized simultaneously. Two-fifths of each amplification mixture was separated on agarose gels and analyzed by blot hybridization.

Sequences of PCR primers. Oligonucleotide primers were as follows: Jβ (5′-CTCCTCATCCTATGGCAC-3′); CH (5′-GCAGATCTCTGTTTTTGCCT CCG-3′); and β-actin (5′-GTGGGCCGCTCTAGGCACCAA-3′, 5′-CTCTTT GATGTCACGCACGATTTC-3′).

RESULTS

Experimental design. Previous analysis of V(D)J rearrangement within transgenes composed of an upstream TCRβ variable gene region linked to a downstream IgH Cμ constant gene region identified a recombination enhancement activity to a 1,100-bp DNA fragment (hereafter referred to as Eμ1100 WT) containing the Eμ enhancer (18). To define the sequences responsible for this activity, we devised similar transgenic experiments using modified recombination substrates. Assuming that these sequences are located within the transcriptional enhancer, we designed a series of related constructs which replaced the Eμ1100 WT fragment in the original TCRβ-IgH Cμ hybrid with various Eμ fragments (Fig. 1). Besides Eμ700 WT, the other variants lacked several regulatory elements due to either point mutations (Eμ700 $E3^-E4^-$) or specific deletions (Eμ300 WT, Eμ460 WT, and Eμ Es^-O^-) within the enhancer region. These modifications have been previously shown to affect moderately (Eμ700 $E3^-E4^-$ and Eμ300 WT) or more severely (Eμ460 WT and Eμ Es^-O^-) the *cis* activation of heterologous promoters in B-lineage cells, as defined by chloramphenicol acetyltransferase assay analysis (Fig. 1B and legend). Insert DNA derived from each construct was microinjected into fertilized mouse eggs to produce transgenic mice. For each construct, at least two transgenic lines were established by crossing independent founders with wild-type animals (Table 1). The individual lines are hereafter designated according to the Eμ variant and the number of transgene copies carried in the genome of the transgenic animals; for example, transgenic mice in the Eμ700 WT:14 line carry 14 copies of the Eμ700 WT recombination substrate. As control, we used transgenic mice bearing 14 copies of the Eμ1100 WT substrate (18).

Recombinational activity of the transgenic substrates. Site-specific rearrangements within the microinjected substrates were first assayed by Southern blot analysis as previously described (9, 18). Briefly, genomic DNA from lymphoid (thymus, spleen, lymph nodes, and bone marrow) and nonlymphoid (kidney and skin) tissues of 4-week-old transgenic mice was digested with the appropriate restriction endonuclease(s) and hybridized to probes specific for the various regions present in the constructs. Substrate rearrangements were inferred from the differential labeling of fragments of predicted size in DNA from lymphoid and nonlymphoid tissues. Analysis of several animals carrying the same construct from independent transgenic lines gave consistent results. Figure 2 shows representative results (lines Eμ1100 WT:14, Eμ700 WT:15, Eμ460 WT:18, Eμ700 $E3^-E4^-$:18, Eμ300 WT:18, and Eμ Es^-O^-:22) obtained from the digestion of genomic DNA with *Bgl*II and hybridization with a Jβ1 probe. As expected, a 12-kb *Bgl*II fragment encompassing the endogenous Jβ1 gene segment in a germ line configuration as well as a 6.0-kb fragment corresponding to the unrearranged transgene (Fig. 2B; compare lanes 2 and 3) were detected in kidney DNA from a transgenic control mouse, Eμ1100 WT:14. Thymus DNA from the same mouse gave additional labeled fragments of 10.5 and 7.0 kb representing substrate DJ and VDJ rearrangements, respectively (lane 1); the endogenous fragment hybridized less intensively in the thymus as a result of TCRβ rearrangements in most thymocytes (57). Because of the related structures of all of the microinjected constructs, similar profiles of hybridization (if substrate rearrangements did occur) could be expected when transgenic tissues from the corresponding lines were

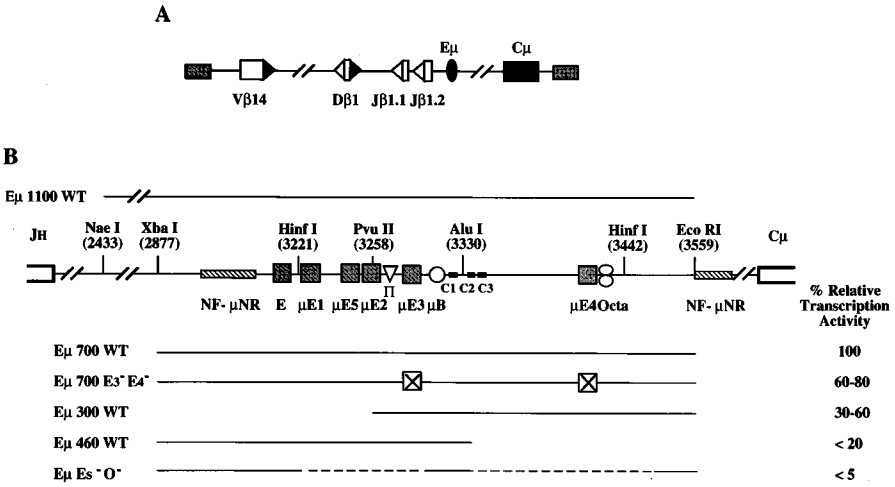

FIG. 1. Structures of the recombination substrates. (A) General structures of the microinjected inserts. The TCRβ V, D, and J segments are represented by open boxes, their flanking recombination signal sequences are represented by shaded (23-bp spacer) or open (12- or 13-bp spacer) triangles, the IgH Cμ exons are represented by shaded boxes, and the cosmid sequences are represented by hatched boxes. Eμ, location of the enhancer fragment. (B) Variants of the Eμ enhancer. A schematic diagram of the Eμ regulatory region is represented. The line indicates the large J_H-Cμ intron of the IgH gene locus, and the symbols indicate the various Eμ elements (as specified in reference 39). The locations of the Eμ variants used in this study and of the Eμ1100 WT fragment used previously (18) are indicated below and above the diagram, respectively. The line indicates wild-type sequences, the crossed squares represent point mutations within the μE3 and μE4 elements, and the dotted lines show deletions in mutant Eμ Es^-O^-. The Eμ variants are named according to their approximate sizes and structures (see text). The percentage of transcriptional activity of each variant relative to that of the Eμ700 WT enhancer (as determined by chloramphenicol acetyltransferase assays after transient transfection into B-lymphoid cells) is indicated (Eμ700 $E3^-E4^-$, Eμ300 WT, and Eμ460 WT, percentage of activation of a c-*fos* promoter [36, 41]; Eμ Es^-O^-, percentage of activation of a simian virus 40 promoter [33]).

analyzed (Fig. 2B, legend). Indeed, bands corresponding to the unrearranged transgenes as well as the predicted DJ- and VDJ-sized substrate fragments were detected in thymuses from transgenic mice in the Eμ700 WT and Eμ460 WT lines (lanes 4 and 7). Substrate DJ rearrangements were also evident in spleens, lymph nodes, and bone marrow from the same animals (except in the bone marrow from the Eμ460 WT mice [see below]) and were absent in the kidneys and skin (lanes 5, 6, 8, and 9 and data not shown). Additional faint hybridizing fragments, which most likely correspond to minor types of V(D)J recombinase-mediated rearrangements within the transgenes (18), were occasionally detected in the lymphoid tissues (lane 7). In contrast, when the lymphoid and the non-lymphoid tissues from transgenic mice in lines Eμ700 $E3^-E4^-$, Eμ300 WT, and Eμ Es^-O^- were compared, only the endogenous and/or transgene unrearranged fragments could be detected (lanes 10 to 12, 13 to 15, and 16 to 18). Note that the hybridization profile of the endogenous 12-kb *Bgl*II fragment in thymus indicated that recombination of the TCRβ gene occurred in thymocytes from all the transgenic mice.

TABLE 1. Transgenic mouse lines[a]

Enhancer fragment[b]	Transgene copy no./ diploid genome[c]
Eμ700 WT (4)	14, 15, 20, 25
Eμ700 $E3^-E4^-$ (2)	3, 18
Eμ300 WT (4)	2, 18, 19, 20
Eμ460 WT (2)	18, 20
Eμ Es^-O^- (3)	8, 14, 22

[a] Transgenic mouse lines were established from independent founders and maintained by crosses with normal (C57BL/6 × CBA/J)F_1 mice; transgenic animals in the various lines were shown to carry mostly intact copies of the transgene, integrated in a head-to-tail configuration at a single site.

[b] Variants of Eμ within the microinjected inserts. The number of transgenic lines produced with each type of insert is in parentheses.

[c] Determined in each line by densitometric analysis of autoradiograms from Southern blots of transgenic kidney DNA and comparison between fragments containing either the substrate or the endogenous Jβ1.1 gene segment.

Using quantitative DNA PCR assays to analyze V(D)J recombination within the reporter transgenes (9), we confirmed the presence of high levels of substrate rearrangement in the lymphoid tissues from all the Eμ700 WT and Eμ460 WT lines (except in the bone marrow from the latter) but not in those tissues from the Eμ700 $E3^-E4^-$, Eμ300 WT, and Eμ Es^-O^- lines. Indeed, with this sensitive technique, we found no rearrangement in the tissues from three Eμ Es^-O^- lines, one Eμ700 $E3^-E4^-$ line, and three Eμ300 WT lines, although we did find low levels of rearrangement in the lymphoid tissues from one Eμ700 $E3^-E4^-$ line and one Eμ300 WT line (data not shown). Altogether, these results led us to conclude that the Eμ700 WT and Eμ460 WT fragments can efficiently stimulate recombinase-mediated rearrangements within the reporter transgenes, whereas the Eμ700 $E3^-E4^-$, Eμ300 WT, or Eμ Es^-O^- fragments cannot.

Characteristics of the Eμ700 WT- and Eμ460 WT-mediated substrate rearrangements. In previous analyses of mice carrying the Eμ1100 WT transgenes, substrate DJ rearrangements were found in both B and T cells, whereas substrate VDJ rearrangement was found preferentially in T cells (18). Presumably, a recombinational Eμ element active in the B- and T-cell lineages initiates Dβ to Jβ rearrangements within the transgenic substrate, while a separate element provides T-cell-specific control of complete (Vβ to DβJβ) variable region gene assembly. This difference in the pattern of substrate rearrangement may be recapitulated in the Eμ700 WT transgenic mice, as we found lower levels of substrate VDJ rearrangement than of substrate DJ rearrangement specifically in the lymphoid tissues composed of a significant proportion of B cells such as spleen and bone marrow cells (Fig. 2B, lane 5; Fig. 3A, lane 4). In the spleens and bone marrow of the Eμ460 WT lines, however, substrate DJ and VDJ rearrangements were both very low (Fig. 2B and 3A, lanes 8), suggesting that Dβ-to-Jβ as

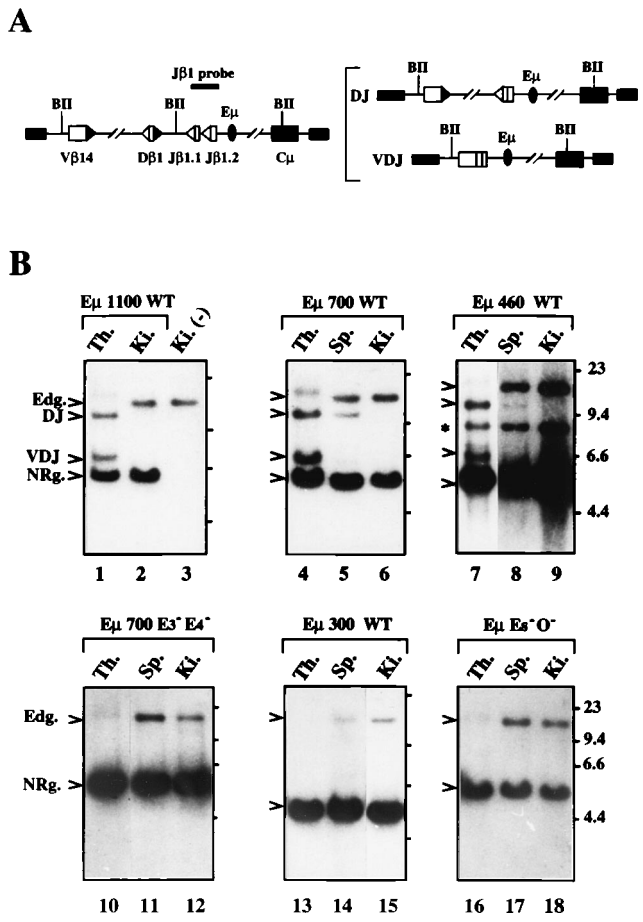

FIG. 2. Analysis of the microinjected constructs in the transgenic mouse tissues. (A) Partial restriction endonuclease map (BII, *Bgl*II) of the microinjected inserts (left) and of the transgenes following potential site-specific DJ and VDJ rearrangements (right; only joins to the Jβ 1.2 gene segment are represented); the location of the Jβ1 probe used in Southern blot analysis is indicated. In Eμ1100 WT transgenic mice, the Jβ1 probe labels the following *Bgl*II substrate fragments: 10.5 kb (DJ), 7.0 kb (VDJ), and 6.0 kb (unrearranged); in the other transgenic mice, the corresponding fragments are shortened by 0.45 kb (Eμ700 WT and Eμ700 $E3^-E4^-$), 0.65 kb (Eμ460 WT), 0.85 kb (Eμ300 WT), and 0.7 kb (Eμ Es^-O^-). (B) Ten micrograms of genomic DNA from tissues of 4-week-old transgenic mice of lines Eμ1100 WT:14, Eμ700 WT:15, Eμ460 WT:18, Eμ700 $E3^-E4^-$:18, Eμ300 WT:18, and Eμ Es^-O^-:22 and of a wild-type animal [(−)] was digested with *Bgl*II and assayed by Southern blot analysis for hybridization to the ^{32}P-labeled Jβ1 probe. Th., thymus; Ki., kidney; Sp., spleen. The positions of the *Bgl*II fragments containing the endogenous Jβ1 gene segment (Edg.), the unrearranged substrate (NRg.), and the DJ and VDJ substrate rearrangements are indicated. Stars indicate truncated copies of the transgene in line Eμ460 WT:18; for this line, the exposure times were varied to optimize visualization of substrate rearrangements in the spleen. Fragment sizes of *Hin*dIII-digested λ phage DNA are indicated in kilobases on the right.

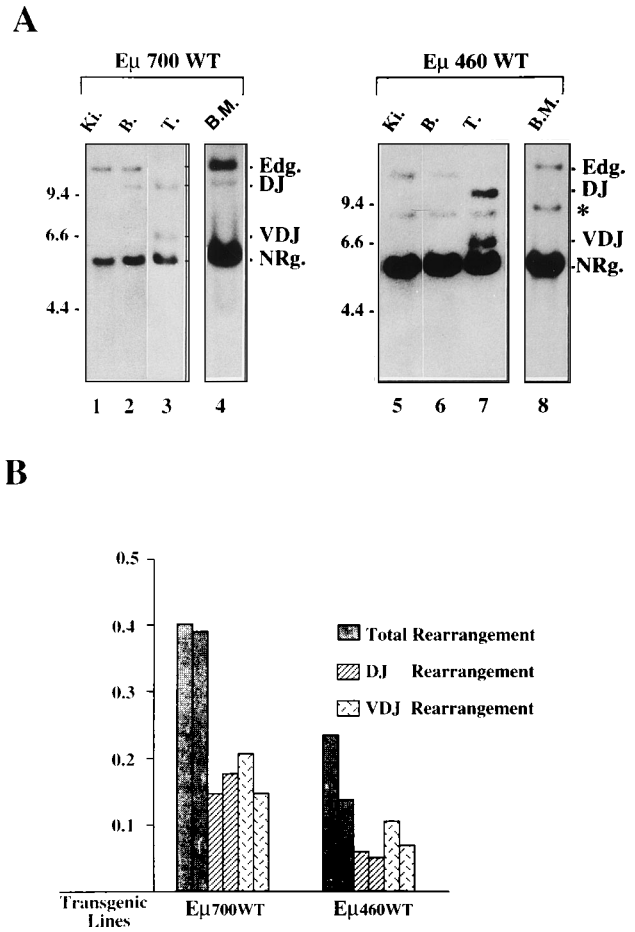

FIG. 3. Characteristics of substrate rearrangements within the Eμ700 WT and Eμ460 WT transgenes. (A) Genomic DNA from tissues and lymphoid cell populations in the Eμ700 WT:15 and Eμ460 WT:18 transgenic mouse lines was processed as described in the legend to Fig. 2. Ki., kidney; B., peripheral B-enriched cells; T., peripheral T-enriched cells; B.M., bone marrow. FACS analyses of the enriched cell populations were as follows: Eμ700 WT:15, T (90% $CD3^+$/6% Ig^+) and B (92% Ig^+/4% $CD3^+$); Eμ460 WT:18, T (96% $CD3^+$/3% Ig^+) and B (89% Ig^+/6% $CD3^+$). (B) Graphic representation of substrate total, DJ, and VDJ rearrangements in thymuses from transgenic mice in the Eμ700 WT:15/25 and Eμ460 WT:18/20 lines. Percentages were determined following densitometry analysis of Jβ1-hybridized filters (see Materials and Methods). The bars represent average percentage values from independent transgenic lines carrying the same substrate; differences between the values of substrate total rearrangement versus the sum of substrate DJ plus VDJ rearrangements most likely reflect the presence of minor types of V(D)J recombinase-mediated rearrangements within the transgenes.

well as Vβ-to-DβJβ rearrangement events of the Eμ460 WT transgenes did not proceed efficiently in the B cells. To verify this point, we purified peripheral B and T lymphocytes from the Eμ700 WT:15 and Eμ460 WT:18 transgenic mice and analyzed substrate rearrangements in each of these two cell populations. Equivalent amounts of substrate DJ rearrangement were found in DNA from the B- or T-cell-enriched populations in the Eμ700 WT:15 line, whereas VDJ joins were detected only in the T-enriched population (Fig. 3A, lanes 2 and 3). In contrast, in the Eμ460 WT:18 line, neither DJ nor VDJ fragments could be detected in the B-enriched cells, while high levels of both rearranged fragments were present in the T-enriched cells (lanes 6 and 7). We conclude that Eμ700 WT, like Eμ1100 WT, stimulates Dβ-to-Jβ joins in both T- and B-cell lineages, whereas the recombinational activity of Eμ460 WT is restricted to the T-cell lineage.

To compare the levels of transgene recombination, we determined the percentages of total, DJ, and VDJ substrate rearrangements in transgenic thymuses of the Eμ700 WT and Eμ460 WT lines following densitometric analyses of Southern blot autoradiograms. On average, the percentages obtained in the Eμ700 WT lines (40% total, 17% DJ, and 18% VDJ substrate rearrangements; Fig. 3B) were comparable to those observed in similar analyses of transgenic thymuses carrying the Eμ1100 WT construct (15). In comparison, lower levels of rearrangements were obtained in the Eμ460 WT lines (averages of 19, 5.5, and 9.5%, respectively). It is of note that, as was

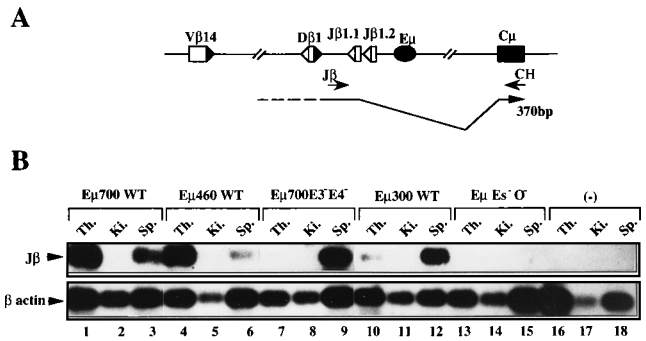

FIG. 4. RNA PCR analysis of Jβ transcription in the transgenic mouse tissues. (A) Diagram of the PCR assay. The structures of the Jβ germ line transcripts and the positions of the Jβ and CH primers in the recombination substrates are indicated. Rearrangements involving the Jβ segments result in a loss of the Jβ primer sequences; because primers which crossed intron-exon boundaries were used, contaminating DNA could not be amplified by PCR. (B) RNA PCR analysis of Jβ (top) and of β actin (bottom) transcripts in transgenic (lines Eμ700 WT:15, Eμ460 WT:18, Eμ700 E3$^-$E4$^-$:18, Eμ300 WT:18, and Eμ Es$^-$O$^-$:22) or wild type mouse tissues [(−)]. Total RNA (1 ng) of each tissue was used for the PCR reaction; the amplified cDNA products were then run on agarose gels, transferred onto nylon membranes, and hybridized with probes internal to the amplified fragments. Th., thymus; Ki., kidney; Sp., spleen.

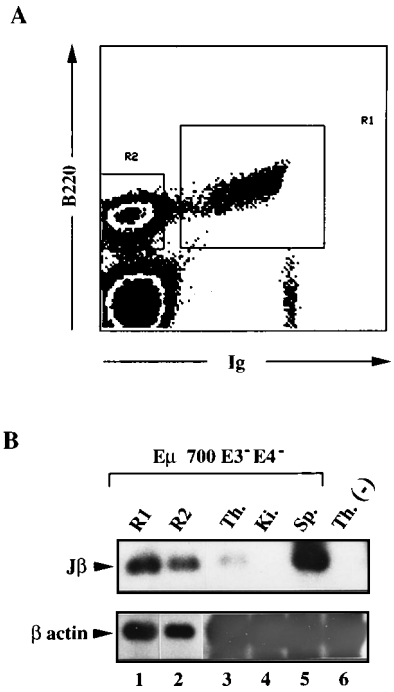

FIG. 5. RNA PCR analysis of Jβ transcription in B-lineage cells from the Eμ700 E3$^-$E4$^-$:18 line. (A) FACS analysis of bone marrow cells from Eμ700 E3$^-$E4$^-$:18 transgenic mice. Cells were stained with fluorescein isothiocyanate-conjugated anti-Ig (H+L) F(ab′)$_2$ fragment and with phycoerythrin-conjugated anti-B220 MAb. The cells in R1 (B220$^+$ sIg$^+$) and R2 (B220$^+$ sIg$^-$) were sorted; they represented 6.5 and 15.3% of total bone marrow cells, respectively. (B) RNA PCR analysis of Jβ and β actin transcripts was done as for Fig. 4 except that 200 R1 and R2 sorted cells were used. Analysis of tissues (thymus [Th.], spleen [Sp.], and kidney [Ki.]) from these animals and of thymus from a wild-type mouse [(−)] is also shown.

previously observed in the Eμ1100 WT-containing transgenes (18), the percentages of rearrangement in independent lines carrying the same construct were roughly equivalent, indicating that the recombinational activity of the Eμ700 WT and Eμ460 WT fragments does not depend on the number of transgene copies and integration site. Thus, the results show that the recombinational activity of Eμ700 WT is comparable with that of Eμ1100 WT, while Eμ460 WT is two- to threefold less active in promoting substrate rearrangement. Therefore, among the various enhancer fragments tested in this study, only Eμ700 WT can effect the cell type and the level of V(D)J recombination previously defined by the Eμ1100 WT transgenes.

Transcriptional activities of the transgenic substrates. To analyze the influence of the Eμ mutations on expression of the unrearranged transgenes, we used a specific RNA PCR assay exploiting the TCRβ-IgH Cμ hybrid structure of our constructs (Fig. 4A). The chosen Jβ and CH primers amplify a 370-nucleotide fragment which corresponds to the germ line transcripts (hereafter referred to as Jβ transcripts) initiating upstream of the unrearranged Jβ1.1 segment and spliced to the first Cμ exon in the transgenes; by limiting the number of amplification cycles, the assay can be used to derive quantitative information (9). Analyses of RNA from thymuses, spleens, and kidneys carrying the same substrate in independent transgenic lines gave consistent results. Figure 4B shows representative results obtained for tissues from a transgenic mouse from either the Eμ700 WT:15, Eμ460 WT:18, Eμ700 E3$^-$E4$^-$:18, Eμ300 WT:18, or Eμ Es$^-$O$^-$:22 line or from a wild-type mouse. As expected, Jβ transcripts were not detected in non-transgenic tissues (lanes 16 to 18). Moreover, they were not detected in the Eμ Es$^-$O$^-$ transgenic tissues (lanes 13 to 15). However, high levels of Jβ transcripts were found in transgenic thymuses and/or spleens (but not in kidneys) for all other recombination substrates, including (i) in the thymuses and spleens from the Eμ700 WT transgenic mice (lanes 1 to 3), (ii) predominantly in the thymuses from the Eμ460 WT transgenic mice (lanes 4 to 6), and (iii) predominantly in the spleens from the Eμ700 E3$^-$E4$^-$ and Eμ300 WT transgenic mice (lanes 7 to 9 and 10 to 12, respectively). When the same RNAs were tested in a separate PCR assay, we found similar profiles of substrate Vβ germ line transcripts (data not shown). Moreover, Northern (RNA) blot analyses indicated that high levels of transgene-derived, Iμ-related transcripts (37) are produced in the thymuses of the Eμ700 WT and Eμ460 WT transgenic mice but not in those of the Eμ700 E3$^-$E4$^-$, Eμ300 WT, and Eμ Es$^-$O$^-$ transgenic mice (15). Assuming that the levels of transcription in the spleen and thymus reflect those in B and T cells (the percentages of B and T cells in the spleen are higher and lower, respectively, than in the thymus [27]), these results are consistent with (i) Eμ700 WT activating transgene expression in both T and B cells, (ii) Eμ460 WT activating transgene expression preferentially in T cells, and (iii) Eμ700 E3$^-$E4$^-$ and Eμ300 WT activating transgene expression preferentially in B cells. We have verified that Jβ transcripts are produced at distinct developmental stages within the B-cell lineage in the Eμ700 E3$^-$E4$^-$:18 transgenic mice by performing RNA PCR analysis of purified, CD45 receptor (B220)/sIg-sorted cells from transgenic bone marrow (Fig. 5). Significantly, we found that levels of Jβ transcription are similar in differentiating (B220$^+$ sIg$^-$) and mature (B220$^+$ sIg$^+$) B cells (Fig. 5B; compare lanes 1 and 2). Because the former population is known to include a majority of cells expressing the recombinase-activating genes Rag-1 and Rag-2 (see reference 38 and references therein), these data strongly suggest that transgene expression in the Eμ700 E3$^-$E4$^-$:18 transgenic mice occurs from V(D)J recombinase-positive stages onward within the B lineage.

Our interpretations of the transcriptional data with respect to transgene expression in T- and B-lineage cells are summarized in Table 2. Thus, transgene expression appears to occur in an enhancer-dependent fashion, with the structure of the

TABLE 2. Transgene expression and recombination in T- and B-lymphoid cells

Transgenic lines[a]	T cells		B cells	
	Germ line transcription[b]	V(D)J recombination[c]	Germ line transcription[d]	V(D)J recombination
Eμ700 WT	High	+	High	+
Eμ460 WT	High	+	Low	−
Eμ700 $E3^{-}E4^{-}$	Low	−	High	−
Eμ300 WT	Low	−	High	−
Eμ $Es^{-}O^{-}$	—	−	—	−

[a] For each type of Eμ variant, at least two independant transgenic lines carrying different copy number were analyzed and gave identical results.
[b] High and low refer to PCR amplification of high and low levels of substrate Iμ, Jβ, and Vβ germ line transcripts in thymus. —, PCR products were not detected.
[c] Presence (+) or absence (−) of substrate DJ rearrangement in Southern analysis of peripheral T- and B-enriched cells and/or of lymphoid tissues.
[d] High and low refers to PCR amplification of high and low levels of substrate Jβ and Vβ germ line transcripts in spleen. —, PCR products were not detected.

enhancer correlating directly with the cell type (T or B) expression of the transgene. Furthermore, a comparison between the transcriptional and recombinational activities conferred by the individual enhancer fragments revealed that these two processes are uncoupled in the B cells from the Eμ700 $E3^{-}E4^{-}$ and Eμ300 WT transgenic mice, in which both of the corresponding fragments can efficiently activate germ line transcription but not V(D)J recombination of the microinjected constructs; it is worth noting that this was observed whether high-copy-number (i.e., Eμ700 $E3^{-}E4^{-}$:18 and Eμ300 WT:18) or low-copy-number (i.e., Eμ700 $E3^{-}E4^{-}$:18 and Eμ300 WT:2) lines were used.

CpG methylation of the transgenic substrates. The activation of Ig or TCR gene expression has been associated with changes in the CpG methylation pattern at these loci (4, 7, 43, 59). In addition, recent studies have linked the degree of DNA methylation to the V(D)J rearrangement potential of various types of recombination substrates (12–14, 24, 28). To analyze whether transgene methylation can be influenced by the associated enhancer, we used the methylation-sensitive endonuclease *Hha*I in a Southern blot analysis of transgenic tissues from the various lines. As shown in Fig. 6A, *Hha*I sites are located in several regions of the recombination construct, including one site 5′ of Vβ, one 5′ of Dβ, and one 3′ of Eμ. The level of CpG methylation at each site was analyzed in the various transgenes by using appropriate restriction enzyme cleavages and hybridization with specific probes (Dβ 3′ and μ800 probes); for each construct, at least two transgenic mice from independent lines were studied. Figures 6B and C show representative results for transgenic tissues from mice in the Eμ700 WT:15, Eμ460 WT:18, Eμ300 WT:18, and Eμ700 $E3^{-}E4^{-}$:18 lines. Analysis of the Eμ $Es^{-}O^{-}$ lines gave results that were similar to those obtained with the Eμ700 $E3^{-}E4^{-}$ and Eμ300 WT lines (data not shown).

In *Bgl*II-*Eco*RI-*Hha*I-digested thymus DNA, two hybridizing fragments were generally detected with the Dβ 3′ probe (Fig.

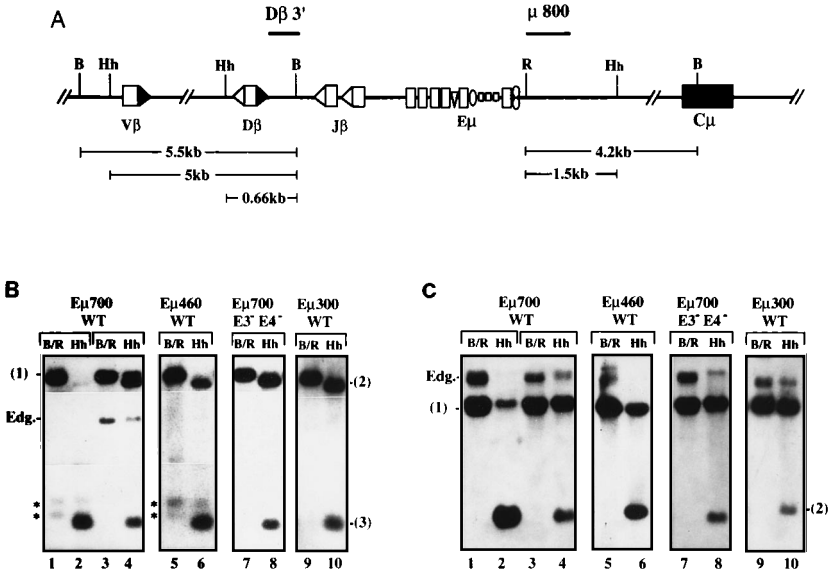

FIG. 6. Methylation status of the recombination substrates. (A) Experimental strategy. The locations of the relevant restriction endonuclease sites (B, *Bgl*II; R, *Eco*RI; Hh, *Hha*I) and probes used to analyze CpG methylation within the transgenic miniloci are indicated. (B and C) Southern blot analysis of transgenic mouse lines Eμ700 WT:15, Eμ460 WT:18, Eμ700 $E3^{-}E4^{-}$:18, and Eμ300 WT:18. Genomic DNA (20 μg) from thymus (lanes 1, 2, and 5 to 10) or kidney (lanes 3 and 4) was digested with *Bgl*II and *Eco*RI and then further digested (10 μg) with *Hha*I. *Bgl*II-*Eco*RI (B/R)- and *Bgl*II-*Eco*RI-*Hha*I (Hh)-restricted DNAs were separated by agarose gel electrophoresis, transferred onto nylon membranes, and hybridized with the Dβ 3′ (B) or μ800 (C) probe. Edg., endogenous *Bgl*II (B) or *Eco*RI-*Bgl*II (C) fragments. In panel B, the endogenous fragments are not visible in the thymus because of TCRβ rearrangements in most thymocytes. Numbers in parentheses correspond to the predicted substrate fragments: in panel B, the 5.5-kb *Bgl*II fragment (1) and the 5- or 0.66-kb *Hha*I-*Bgl*II fragment (2 or 3); in panel C, the 4.2-kb *Eco*RI-*Bgl*II fragment (1) and the 1.5-kb *Eco*RI-*Hha*I fragment (2). Asterisks in panel B indicate the linearized fragments produced by *Bgl*II digestion of the circles DNA yielded by substrate DJ rearrangements in the thymus of the Eμ700 WT:15 and Eμ460 WT:18 mice.

6B, lanes 2, 6, 8, and 10). These fragments correspond to *Bgl*II-*Hha*I fragments encompassing either the substrate unrearranged Dβ gene segment alone (fragment 3, 0.66 kb) or both substrate unrearranged Vβ and Dβ segments (fragment 2, 5 kb). The overall disappearance of the 5.5-kb *Bgl*II fragment (fragment 1, also comprising both Vβ and Dβ segments) indicated that the 5′ Vβ *Hha*I site in the substrate is digested completely, and therefore uniformly demethylated, in transgenic thymuses. Moreover, variations in the relative intensity of fragment 3 compared with that of fragment 2 implied that the 5′ Dβ *Hha*I site is differentially methylated among the various transgenic lines. To estimate the extent of methylation at this site, we calculated the fragment 3/fragment 2 ratio following densitometric analyses of the autoradiograms. These analyses indicated that the 5′ Dβ *Hha*I site in the substrate is hypomethylated in thymuses of the Eμ700 WT mice (for example, the ratio between fragment 3 and fragment 2 in the Eμ700 WT:15 line is ~8:1), partially demethylated in the Eμ460 WT lines (the ratio in the Eμ460 WT:18 line is ~2:1), and relatively methylated in the Eμ700 $E3^-E4^-$, Eμ300 WT, and Eμ Es^-O^- lines (the ratio in both the Eμ700 $E3^-E4^-$:18 and Eμ300 WT:18 lines is ~1:3). Conversely, similar analysis of transgenic kidneys indicated a relative hypermethylation of the 5′ Dβ *Hha*I site, at roughly comparable levels among the various constructs (lane 4; only the kidney from a Eμ700 WT:15 transgenic mouse is shown; the ratio is ~1:3); note that CpG methylation in kidney extends to the 5′ Vβ *Hha*I site, as shown by the presence of a low level of fragment 1.

Hybridization of the same blots with the μ800 probe allowed the analysis of the 3′ Eμ *Hha*I site, located downstream of Eμ in the integrated substrates. In the thymus, this analysis (Fig. 6C, lanes 2, 6, 8, and 10) suggests a profile of CpG methylation which is very similar to that of the 5′ Dβ *Hha*I site. This includes hypomethylation in the Eμ700 WT lines, partial demethylation in the Eμ460 WT lines, and relative hypermethylation in the Eμ700 $E3^-E4^-$, Eμ300 WT, and Eμ Es^-O^- lines. For example, densitometric analysis indicated a ~8:1 ratio of the intensity of the *Eco*RI-*Hha*I 1.5-kb fragment 2 to that of the *Eco*RI-*Bgl*II 4.2-kb fragment 1 in the Eμ700 WT:15 line, a ~3:2 ratio in the Eμ460 WT:18 line, and a ~1:4 ratio in both the Eμ700 $E3^-E4^-$:18 and Eμ300 WT:18 lines. Again, the 3′ Eμ *Hha*I site in the transgenes appeared relatively hypermethylated in the kidney (lane 4; the ratio in the Eμ700 WT:15 line is ~1:4). Together, the results indicate that sequences within the Eμ enhancer somehow influence the level of CpG methylation of the surrounding regions in chromosomal integrated genes. Significantly, in the thymus, hypermethylation at the 5′ Dβ and 3′ Eμ *Hha*I sites correlates with the absence of substrate rearrangement and expression, whereas conversely, demethylation at the same sites correlates with the presence of substrate rearrangements along with the production of germ line transcripts (see above). Since Eμ460 WT has reduced activity for recombination compared with Eμ700 WT, it is also noteworthy that the Eμ460 WT transgenes are less demethylated than the Eμ700 WT transgenes. Whether this difference reflects demethylation of only a few copies of the tandemly integrated Eμ460 WT transgenes or demethylation of the whole transgenic array in a lower percentage of the thymic cells remains to be determined.

DISCUSSION

***cis*-acting components of the Eμ transcriptional enhancer activate V(D)J recombination in a transgenic substrate.** Our studies demonstrate unequivocally that the *cis*-acting components of the Eμ transcriptional enhancer activate V(D)J recombination of associated variable gene segments in chromosomally integrated genes. Thus, within a transgenic recombination substrate, Eμ700 WT promotes V(D)J recombinase-mediated rearrangements of TCRβ V, D, and J segments with a pattern superimposable onto that previously observed within transgenes containing a longer Eμ1100 WT fragment. Conversely, substrate rearrangements are variously affected within transgenes carrying diverse truncations of the Eμ enhancer (i.e., Eμ460 WT, Eμ300 WT, or Eμ Es^-O^-). Most significantly, point mutations within μE3 and μE4, two E-box motifs present in the Eμ700 WT fragment, impair recombination of the reporter transgenes. Because lines carrying roughly equivalent numbers of transgenic copies were compared (Fig. 2), it is unlikely that the differences observed are influenced by a multimerization of transcription binding sites in the tandem transgenic array. Whether the μE3 and/or μE4 sequences, in possible conjunction with other E-box motifs (see below), truly account for the V(D)J recombinational activity of Eμ at the endogenous IgH gene locus remains to be confirmed. However, this proposition would be in agreement with the presence of E-box motif homologies in other lymphoid cell-specific enhancers endowed with a recombinational activity (9, 31, 34, 35, 60).

The regulatory sequences in Eμ700 WT stimulate efficiently substrate rearrangements in B- and T-lymphoid cells. However, the μE3 and/or μE4 motifs are not sufficient to support this activity given that Eμ300 WT, in which those two motifs are intact, is ineffective in V(D)J recombination. Thus, an additional element(s) essential for recombination must be located in the Eμ region upstream of Eμ300 WT, an assumption also supported by the recombinational activity of the Eμ460 WT fragment. Nonetheless, because rearrangements are less efficient in the Eμ460 WT lines than in the Eμ700 WT lines, the Eμ upstream element(s) cannot account for full enhancer activity either. Our current results point to one or several of the μE sites located 5′ of μE3 (i.e., μE1, μE2, and μE5) as a likely candidate(s). In support of this possibility, preliminary analyses of lymphoid tissues from mice transgenic for a construct carrying the 220-bp *Hin*fI Eμ core fragment extending from sequences 5′ of μE1 to 3′ of μE4/octanucleotide motifs (Fig. 1) indicate that this DNA fragment readily promotes substrate rearrangements (15). In the same line, overexpression of a μE2/μE5-binding transcription factor in stable transfectants resulted in a large induction of Ig D_H-to-J_H rearrangement (54). On the basis of all of these findings, we propose that the V(D)J recombinational activity of the IgH intronic enhancer depends on interactions involving several E-box motifs spanning this element. Studies on transcriptional activation by Eμ enhancer components have affirmed the synergistic action of these motifs (references 39 and 58 and references therein). Therefore, compound *cis*-acting elements made of adjacent E-box motifs might play a dual role during lymphocyte differentiation in regulating both transcription and V(D)J recombination.

The analysis of the Eμ460 WT transgenic mice further illustrates the complex interplay of enhancer elements which dictate the V(D)J recombinational properties of Eμ. The Eμ460 WT fragment allows V(D)J recombinase accessibility to the transgenes, yet substrate rearrangements occur at a lower frequency than those induced by Eμ700 WT. Additionally, Eμ460 WT-mediated rearrangements appear restricted to cells of the T lineage. Taken together, our data therefore suggest that a compound E-box motif element in the 109-bp *Hin*fI-*Alu*I fragment which encompasses μE1, μE5, μE2, and μE3 (Fig. 1) may be sufficient for substrate rearrangements in T cells, whereas interactions involving the same element (or a distinct

element within the same fragment) and additional 3′ Eμ sequences (possibly μE4) may be required for substrate rearrangements in B cells. Differences in the operating characteristics of Eμ recombinational activity in T and B cells are not surprising, considering that endogenous IgH gene rearrangements in T cells occur only at an intermediate stage of thymocyte maturation (after TCRβ genes have been rearranged) and thus are formed independently of those occurring in early B-cell precursors (6). Accordingly, our unpublished analysis of substrate V(D)J recombination during T-cell differentiation has shown that within Eμ700 WT-driven transgenes, most rearrangements are delayed until the late double-negative (i.e., $CD44^-$ $CD25^-$ $CD4^-$ $CD8^-$) stage of thymocyte development (8); this observation suggests that the data obtained from analysis of the TCR-Ig chimeric transgenes may be of general relevance with respect to the recombinational activity of the Eμ enhancer.

The Eμ enhancer and the mechanism of V(D)J recombinational accessibility. Because *cis*-acting motifs of the Eμ transcriptional enhancer appear crucial for the rearrangement of the associated gene segments, one might simply propose that this element interferes with V(D)J recombinational accessibility through its action on transcription. Indeed, in most cases, we do observe a faithful correlation between the levels of substrate germ line expression and recombination (Table 2). Moreover, the recombination-competent Eμ700 WT and Eμ460 WT transgenes produce higher amounts of Jβ transcripts in the thymus than in the spleen (Fig. 5), in agreement with the presence of differentiating lymphocytes active in substrate recombination in the former tissue only. However, our analyses showing B-cell-specific expression of the unrearranged Eμ700 $E3^-E4^-$ and Eμ300 WT transgenes demonstrate that enhancer-mediated V(D)J recombination and transcription can be uncoupled in certain cases, thus putting into question this simple interpretation. Activation of heterologous promoters by the Eμ700 $E3^-E4^-$ and Eμ300 WT fragments when transiently transfected into myeloma cells has already been shown (33, 36). The detection of Jβ transcripts in differentiating ($B220^+$ sIg^-) and mature ($B220^+$ sIg^+) cells from transgenic bone marrow in a Eμ700 $E3^-E4^-$ line demonstrates that the Eμ700 $E3^-E4^-$ fragment is also active in a chromosomally integrated context of normal cells throughout the B lineage, including those expressing V(D)J recombinase activity (38). This is likely to be also true for the Eμ300 WT fragment, considering its ability to activate a c-*fos* promoter when also transiently transfected into recombinase-positive early pre-B- or pre-B-cell lines (40, 41). Therefore, our data are best interpreted by assuming that one regulatory motif or a combination of regulatory motifs present in Eμ700 $E3^-E4^-$ and Eμ300 WT genes (for instance, the π/μA, μB, and/or octanucleotide motifs) drives substrate expression in developing B cells without permitting substrate rearrangements, thus suggesting that transcription, if necessary, is not sufficient per se to induce V(D)J *cis* recombination in recombinase-positive cells. Consequently, the compound E-box motif elements which we define here as being crucial for the recombinational activity of Eμ must operate, at least in part, at a level distinct from transcription. Other experiments also support the lack of an essential mechanistic connection between transcription and V(D)J or Ig-switch recombinations (22, 26, 45, 54).

At which level do the recombinational elements of Eμ operate? One possibility is targeted DNA demethylation. A requirement for *cis*-regulatory elements in tissue- and cell-stage-restricted demethylation of specific genes has been shown by cell transfection studies which examined the role of upstream sequences on demethylation of the α-actin gene in myoblasts (46) and of the κ-chain intronic enhancer on demethylation of the Igκ gene in B cells (32, 42); likewise, our findings that CpG methylation of transgenic Eμ-proximal sequences in the thymus is influenced by mutations in the enhancer indicate that this element is able to affect methylation of the surrounding sequences when assayed in germ line transformation assays. Nonetheless, considering that actively transcribed DNA is usually hypomethylated and that, in B cells, the transcribed Eμ700 $E3^-E4^-$ and Eμ300 WT transgenes are not rearranged, we favor a second scheme, that the recombinational factor brought into play through the μE sites operates independently of demethylation as well. Along this line, the 5′ Dβ and 3′ Eμ *Hpa*I sites within the Eμ700 $E3^-E4^-$ and Eμ300 WT transgenes appear less methylated in the spleen than in the thymus (15). Analyses are in progress to clarify this issue.

Nucleoprotein complexes including clustered sites of enhancers are thought to stimulate gene expression by interacting directly with components of the basal transcription machinery and by initiating factor access to target sites in the nuclear chromatin. Indeed, recent experiments designed to examine the ability of the Eμ region in conferring accessibility upon a linked T7 promoter suggest that a 95-bp Eμ enhancer core can establish localized factor access independently of its chromosomal position; this enhancer-dependent factor access could be uncoupled from an active transcriptional state, although transcription from a nearby promoter was suggested to potentiate this effect (29). Therefore, these properties (i.e., mediation of protein-protein contact and of localized factor access) can account, at least in part, for how the Eμ enhancer modulates V(D)J recombination, assuming that enhancer-mediated transcriptional activation creates the chromosomal alterations that establish general factor access to the chromatin template. At the level of our transgenic model, this would explain why substrate germ line transcription was constantly found in tissues which rearrange the reporter transgenes (reference 9 and this study). However, our data implying that subsets of Eμ components may be sufficient to induce transcriptional activation but not V(D)J recombination of the adjacent genetic regions support a novel concept that, in addition to providing docking sites for factors that dictate transcriptional accessibility, the Eμ enhancer must have some specific function(s) for mediating DNA rearrangement. Such functions may provide specific entry sites for the recombinase machinery and/or allow the formation of a recombinational complex. Ultimately, the recombinational activity of the lymphoid cell-specific enhancer elements may rely on interactions within higher-order nucleoprotein complexes involving *trans*-acting factors with transcriptional and/or structural properties. In reference to our results, it is of interest that the μE3-binding proteins such as TFE-3, USF, and TFEB (3, 10, 19, 25, 49) exhibit not only an ability to activate transcription but also a capacity to induce stable DNA bending (20, 47) or to form tetramers which may hypothetically bind to spatially distinct DNA recognition sites (2, 16, 19).

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