792–798 Nucleic Acids Research, 1994, Vol. 22, No. 5

Sequences affecting the V(D)J recombinational activity of the IgH intronic enhancer in a transgenic substrate

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Received November 24, 1993; Revised and Accepted February 4, 1994

ABSTRACT

The immunoglobulin heavy chain intronic transcriptional enhancer (Eμ) is part of a complex cis-regulatory DNA region which has notably been shown to modulate V(D)J rearrangements of associated variable gene segments. We have used recombination substrates comprised of the Eμ enhancer together with various lengths of additional downstream μ sequences to assess the individual contribution of those sequences to the V(D)J recombinational regulatory activity. Surprisingly, in the absence of large amounts of μ sequences, substrate rearrangements were not detected in Southern blot analyses of the lymphoid tissues from independent transgenic mice, but were readily detectable following transfection into cultured pre-B cells. A short μ segment which includes matrix association regions (MARs) was not sufficient to restore high levels of rearrangements within the reporter transgenes. However, additional experiments demonstrated that the μ sequences are dispensable for V(D)J recombination in transgenic thymuses, implying a suppressive effect exerted by the vector sequences left in the transgenic insert, when they are attached near the Eμ regulatory region. This suppression of V(D)J recombination, which correlates with an hypermethylation of the transgenes, is discussed in view of previously reported transgenic and gene targeting experiments.

INTRODUCTION

Immunoglobulin (Ig) and T cell receptor (TCR) genes are similarly organized into variable and constant regions. In developing (pre-) B and T lymphocytes, Ig or TCR variable regions are assembled from separate V (variable), D (diversity) and J (joining) gene segments by a site-specific recombination system referred to as V(D)J recombinase (1,2). V(D)J recombination events are strictly controlled with respect to cell-type and cell-stage of development. This is thought to involve cis-acting regulatory elements that would modulate the accessibility of the different Ig/TCR gene loci and/or segments to the recombinase activity (3,4).

To characterize the regulatory elements which influence recombinational accessibility, a model system has been previously developed, in which a hybrid (TCRβ variable region/Ig heavy [H] constant μ region) minilocus is introduced into an Abelson murine leukemia virus (A-MuLV) transformed pre-B cell line, or into fertilized mouse eggs to produce transgenic mice (5,6). Notably, it has been shown that the minilocus undergoes lymphoid-specific rearrangements in independent lines of transgenic mice, provided it contains a 1.1 kilobases (kb) fragment from the IgH μ gene region. In view of the correlation between V(D)J recombination and germline transcription of Ig/TCR genes (7,8), it was significant that this fragment includes the IgH intronic transcriptional enhancer (Eμ) (9–12). In accordance with these transgenic studies, targeted deletion or replacement of a DNA segment comprising of the Eμ enhancer core and associated flanking sequences have recently been shown to impair V(D)J recombination events at the IgH gene locus (13,14).

The Eμ enhancer core shows a complex arrangement of several different overlapping stimulatory and negative components (see 15,16 for reviews). Putative regulatory elements have additionally been localized in the Eμ-flanking regions. Thus, matrix association regions (MARs) are present on both sides of Eμ (17). Presumably, the MARs constitute chromosomal loop anchorage elements and may facilitate the DNA–protein interactions required for establishing transcriptional competence (18). Interestingly, DNA-binding sites for putative transcriptional suppressor(s) overlap with the Eμ-associated MARs (19). In addition, nucleotide conservation between mouse and human, remarkably high in the Eμ enhancer, extends to the region comprised of the Eμ 3′ MARs and beyond. Transcription originating heterogeneously from this region has been proposed to contribute to the accessibility of the IgH gene locus for rearrangement (20,21).

The respective roles of the various Eμ-associated and/or Eμ-flanking elements in the control of the V(D)J recombination process, as well as the exact mechanism(s) by which they may contribute to this regulation, remain to be determined. In this study, we have introduced recombination substrates carrying various amounts of Eμ downstream sequences into the genome of transgenic mice or of A-MuLV-transformed pre-B cells. Analysis of substrate assembly demonstrates that V(D)J recombination proceeds efficiently in the absence of all IgH sequences located downstream of the 1.1kb Eμ-containing fragment. However, our results also point out that prokaryotic

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sequences can inhibit substrate recombination when located close to the Eμ regulatory components.

MATERIALS AND METHODS

Cell culture

The derivation, growth, and characteristics of the thymidine kinase negative variant (tk^-) of the 38B9 A-MuLV transformed pre-B cell line have been described previously (22).

Construction of the recombination substrates

The construction of the cVTE, cVTCμ, and cVTECμ substrates have been described previously (6). Substrate cVTEμ800 was constructed by ligating the 820 base pairs (bp) *Eco*RI−*Hinc*II fragment (nucleotides #3560 to #4383 in locus MUSIGCDO7/GENBANK, accession# J00440) into the unique *Eco*RI site of construct cVTE, using *Eco*RI linkers. Within all constructs, the several types of V(D)J recombinase-mediated rearrangements that could theoretically occur by deletion are as described previously (6).

Cell transfection and production of transgenic mice

The constructs were linearized with restriction enzymes *Nru*I and *Pvu*I, which cut into the pMCS cosmid vector (23), yielding inserts cVTE (11.4kb), cVTCμ (20.2kb), cVTECμ (21.4kb), or cVTEμ800 (12.2kb). Insert VTE (8.8kb) was obtained after complete digestion of the cVTE construct with *Eco*RI and partial digestion with *Hind*III. All inserts were purified on agarose gels, followed by passage over Elutip-d columns (Schleicher and Schuell). For cell transfection, 4×10^6 cells were co-electroporated (200V; 960μF) with 25μg of insert plus 2.5μg of linearized pSV2-Neo plasmid (24), using a Gene pulser system (Bio-Rad). Cell transfectants were selected in G418 supplemented media (1mg/ml) and sub-cloned by limiting dilution before analysis.

Microinjection of insert DNA (2μg/ml) into fertilized (C57BL/6×CBA/J) F2 eggs, identification of founder mice, production and characterization of the transgenic lines were carried out as described (6). Three cVTE and four cVTEμ800 independent lines (the 3.12, 3.16, 3.60 and the 4.6, 4.8, 4.20, 4.150 lines) were analyzed carrying 12, 16, or 60 and 6, 8, 20, or >150 transgene copies, respectively. All lines were shown to carry mostly intact copies of the transgene integrated at a single site in a head-to-tail configuration. As controls, we used transgenic mice of the 382, 392 and 1016 lines, carrying 14 and 4 copies of the cVTECμ insert or 20 copies of the cVTCμ insert, respectively (6).

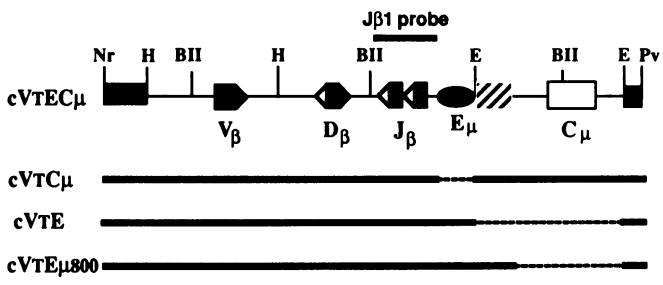

Figure 1. Diagrams of the cVTECμ, cVTCμ, cVTE, and cVTEμ800 substrates. Top panel: partial restriction endonuclease map of recombinant insert cVTECμ. The TCRβ V, D, and J gene segments are represented by shaded boxes, their flanking recombination signal sequences by black (23 bp spacer) or white (13 bp spacer) triangles, the (1.1kb) Eμ-containing fragment by an oval, the (820bp) Eμ-downstream segment (including the Eμ 3′ MARs) by hatched bars, the Cμ exons by an open box, and the cosmid sequences by black boxes; these elements are not drawn to scale. BII: *Bgl*II, E: *Eco*RI, H: *Hind*III; Nr: *Nru*I; Pv: *Pvu*I (not all BII and H sites are shown). The location of the Jβ1 probe used in Southern analyses is indicated. Bottom panel: the sequences absent in the recombinant inserts cVTCμ, cVTE, and cVTEμ800 are indicated by dotted lines; cVTE and cVTEμ800 differ by the presence of 820bp of Eμ-downstream sequences in the latter.

Southern blot analyses

DNA preparations, restriction enzyme digests, agarose gel electrophoresis, DNA blotting procedures, preparation and ^{32}P-labelling of DNA probes, and hybridization procedures were done according to standard protocols. The Jβ1 probe was a 390bp *Xba*I−*Bam*HI fragment containing the unrearranged Jβ1.1 and Jβ1.2 segments from the murine TCRβ gene (25); the Eμ 300 probe was a 300bp *Pst*I−*Eco*RI fragment containing the 3′ region of the mouse IgH intronic enhancer (10). Percentage of DJ, V(D)J, and total substrate rearrangements were determined following densitometric analyses of hybridized filters on a Baf 1000 Imaging Plate Device (Fuji), as described previously (26).

DNA PCR assays

DNA PCR assays to quantitatively analyze substrate rearrangements were as described (26), except for the following modifications: 1) 50ng of purified genomic DNA was used; 2) as a 3′ primer to amplify the cVTE insert, we used an oligonucleotide located upstream of the Eμ core enhancer (i.e. oligonucleotide IgE: 5′-CAGGGACTCCACCAACACCA-3′).

RESULTS AND DISCUSSION

Sequences affecting rearrangement events in a recombination substrate

A recombination substrate comprised of a TCRβ variable minilocus linked to an IgH Cμ constant region gene (Fig.1, cVTECμ) has been shown previously to undergo site-specific rearrangements in lymphoid tissues of transgenic mice (6). Moreover, substrate rearrangements were shown to be dependent on the presence of a cis-acting Eμ-containing DNA fragment because identical transgenes missing this fragment (Fig.1, cVTCμ) were inert for rearrangement. Further studies have demonstrated that the V(D)J recombinational enhancing activity involves the stimulatory components present in the Eμ enhancer core (C.Fernex *et al.*, in preparation).

These results do not preclude whether additional sequences (i.e. besides those in the enhancer core) can affect the rearrangement events within the reporter transgenes. Potential candidates include the regulatory elements in the sequences flanking both sides of Eμ (see above). As a first step to address this issue, we have produced lines of transgenic mice using two related recombination substrates that carry various amounts of m sequences downstream of Eμ (Fig.1: cVTE and cVTEμ800). For each line, substrate rearrangements were initially analyzed by Southern blot assays, using genomic DNA from transgenic tissues, as described previously (6,26); genomic DNA from tissues of transgenic mice carrying the cVTECμ or cVTCμ substrate was used as a control (see Materials and Methods for details on the transgenic lines used in this study). Representative results are reported in figure 2, which shows hybridization of the Jβ1 probe to *Bgl*II−*Eco*RI-digested DNA from a lymphoid (thymus) and a non-lymphoid (kidney) tissue. Thymic DNA from mice carrying the cVTE,

cVTEμ800, or cVTCμ substrate only contains the Jβ1-hybridizing fragments that correspond to the unrearranged transgenes (lanes 5–10), whereas, as expected, thymic DNA from a control mouse carrying the cVTECμ substrate contains additional fragments indicative of substrate DβJβ and Vβ(Dβ)Jβ rearrangements (lanes 1 and 2; these same patterns have also been detected in the spleen and lymph nodes of the individual mice—data not shown). The decrease of the endogenous fragment in thymuses as compared to the corresponding kidneys (due to TCRβ rearrangements in most thymocytes) confirmed the expression of a functional V(D)J recombinase in thymocytes of all transgenic mice. In subsequent studies using quantitative DNA PCR assays to analyze substrate rearrangement (26; see also Materials and Methods), we failed to detect substrate DβJβ or Vβ(Dβ)Jβ joins in transgenic thymus from all the cVTE lines and from two out of four cVTEμ800 lines; with these sensitive assays however, substrate rearrangements were readily detected at low level (less than 5% of those found in the cVTECμ controls) in the two remaining cVTEμ800 lines (Table 1 and data not shown). Taken together, these results indicate that high levels of substrate rearrangements occurred solely in the cVTECμ transgenes; conversely, substrate rearrangements were inefficient not only in the cVTCμ transgenes, but also in the cVTE and cVTEμ800 transgenes as well. Along the same lines, we found no mRNAs transcribed from the cVTCμ, cVTE, and cVTEμ800 transgenes in the thymus, whereas transcription of the cVTECμ transgenes was readily detectable (data not shown; see also 4,6).

The recombination potential of the various substrates was similarly evaluated following electroporation into the A-MuLV-transformed pre-B cell line 38B9tk$^-$. Previous studies have established that introduced TCR Dβ and Jβ (but not Vβ) gene segments can readily be recombined in this cell line (5,27). Cell-clones from individual transfection experiments were isolated as described in Materials and Methods and were analyzed by Southern blot assays. The comparison between parental (38B9tk$^-$) and clone DNAs indicated that most clones had integrated intact copies of the transfected substrate. Representative

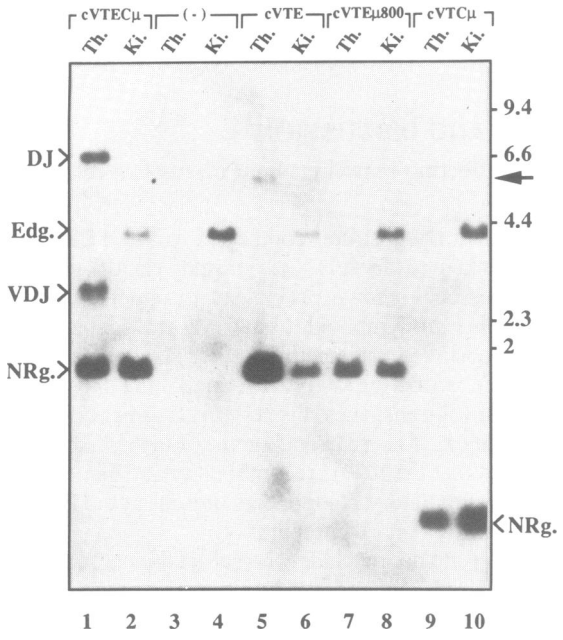

Figure 2. Southern analysis of the cVTECμ, cVTCμ, cVTE, and cVTEμ800 substrates in tissues of transgenic mice. Genomic DNA (10μg) from thymus (Th.) or kidney (Ki.) of 4 week old mice carrying the cVTECμ (line 382), the cVTE (line 3–12), the cVTEμ800 (line 4–8), or the cVTCμ (line 1016) substrate, and of a wild type mouse (−), was digested with *Bgl*II + *Eco*RI and assayed by Southern blot for hybridization to the ^{32}P-labeled Jβ1 probe. The positions of the fragments containing respectively the endogenous unrearranged Jβ1 segment (Edg.: endogenous; 4.3kb), the substrate unrearranged Jβ1 segment (NRg.: non-rearranged; 1.7kb, except in the cVTCμ line, 0.6kb, because of the absence of the Eμ-containing fragment in the corresponding insert), and the DβJβ and Vβ(Dβ)Jβ substrate rearrangements (DJ and VDJ; 6.6kb and 2.7kb, except in the cVTCμ line in which the corresponding fragments would be 5.5kb and 1.6kb, respectively) are indicated. The arrow indicates a truncated copy of the transgene in tissues of the 3–12 line. Fragment sizes of *Hind*III-digested λ phage DNA are indicated in kb on the right.

Table 1. DβJβ substrate rearrangements in the transgenic thymuses

Recombination Substrates	Transgenic Lines	% Rearranged[a] Substrate
cVTECμ	382	100
	392	85
cVTCμ	1016	nd
cVTE	3.12	nd
	3.16	nd
	3.60	nd
cVTEμ800	4.6	nd
	4.8	4
	4.20	2
	4.150	nd

[a] relative percentage of DβJβ rearranged substrate in thymus was determined by densitometric scanning of the substrate rearranged Dβ–Jβ containing fragments on autoradiograms, following DNA PCR assays. The maximum (100%) value was attributed arbitrarily to substrate rearrangements in transgenic thymus of the 382 line; nd: not detected. The amounts of input DNA were normalized by scanning parallel amplifications of a DNA fragment in the RAG-2 gene.

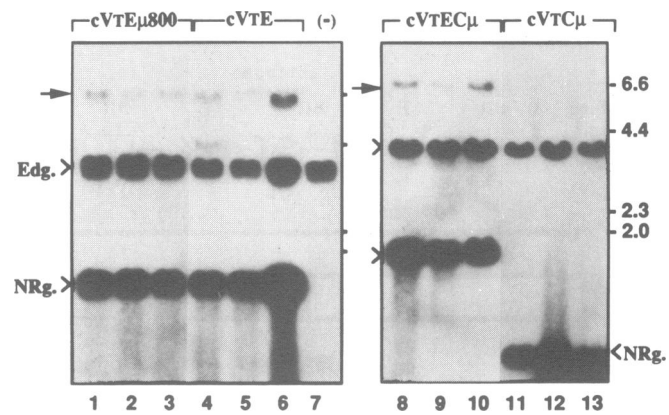

Figure 3. Analysis of the cVTECμ, cVTCμ, cVTE, and cVTEμ800 substrates in transfected cells. Genomic DNA samples from parental 38B9tk$^-$ cells (−) and from independent cell-clones transfected with inserts cVTEμ800, cVTE, cVTECμ, or cVTCμ were analyzed as described in Figure 2. The arrows indicate the 6.6kb submolar fragment carrying substrate DβJβ rearrangements within the cVTEμ800, cVTE, and cVTECμ inserts. The additional submolar fragment seen in lane 4, the size of which is not compatible with site-specific rearrangements within the cVTE insert, is likely to carry aberrant substrate rearrangements, as described previously (5,27).

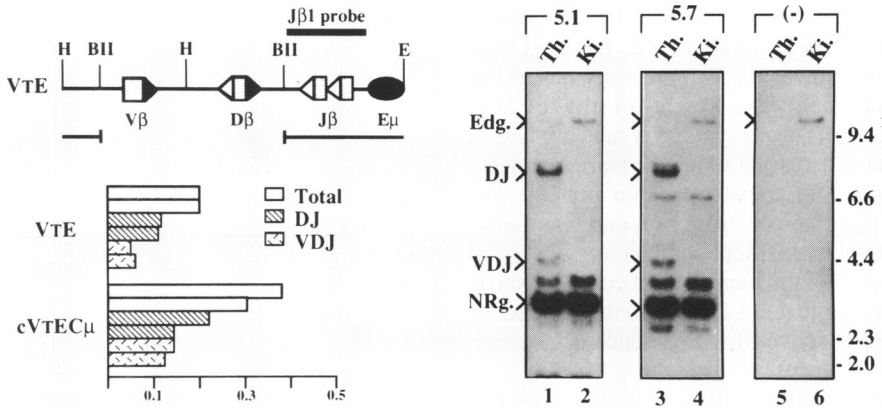

Figure 4. Analysis of the VTE substrate in tissues of the transgenic mice. Left panel. Top: a partial restriction endonuclease map of the VTE insert is arranged as in figure 1. The bars below the diagram indicate the sequences which generate the *Bgl*II unrearranged-fragment (2.9 kb) hybridizing with the Jβ1 probe. Bottom: a graphic representation of substrate rearrangements in transgenic thymus from mice carrying the VTE or cVTECμ inserts. Percentages of DβJβ, Vβ(Dβ)Jβ and total substrate rearrangements were determined following densitometry analysis of Southern blots of genomic DNA from the 5.1 and 5.7 founder mice (carrying the VTE insert) or from a transgenic mouse in the 382 and 392 lines (carrying the cVTECμ insert). Right panel: Jβ1-hybridization of *Bgl*II-digested genomic DNA from thymus and kidney of mice born following microinjection of the VTE insert. The legends are as in figure 2. In the assay, the unrearranged VTE inserts appear as a predominant fragment, 2.9kb in length, junctional between adjacent copies of the transgene in a head-to-tail configuration; DβJβ and Vβ(Dβ)Jβ substrate rearrangements generate *Bgl*II fragments of 7.7kb and 4.1kb, respectively. Other fragments present in both thymus and kidney of the 5.1 and 5.7 mice are likely to correspond to junctional fragments between a few truncated copies of the transgene, between normal copies in opposite orientation, or between transgene and host DNA.

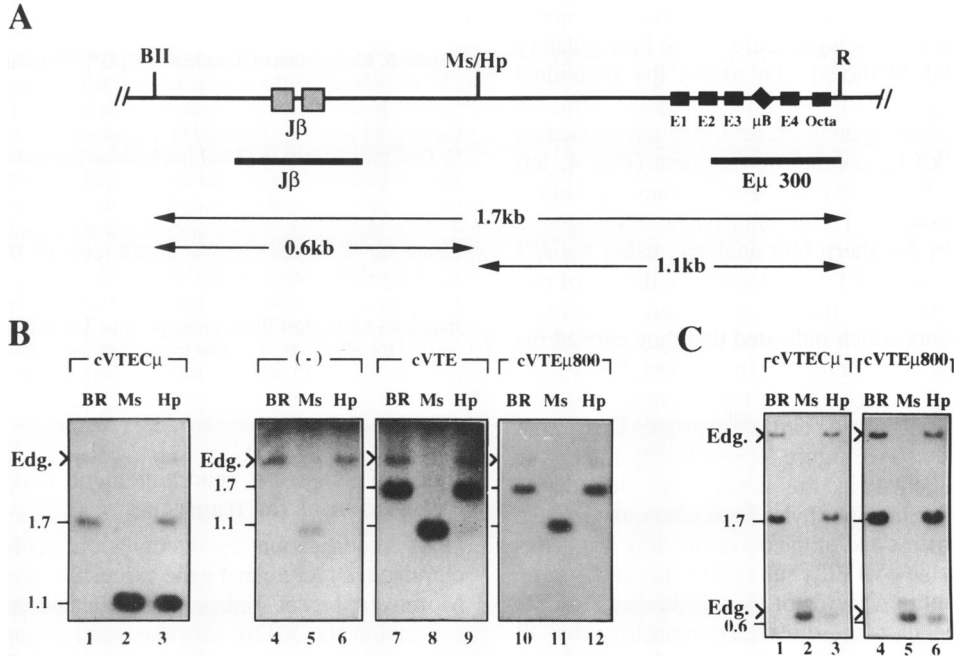

Figure 5. Methylation of dinucleotide CpG$^{\#2435}$ within the integrated substrates. (**A**): partial restriction endonuclease map of the Jβ- and Eμ-containing regions within the recombination substrates; the unrearranged Jβ segments are represented by dotted squares and the stimulatory components of the Eμ enhancer core by black rectangles; BII: *Bgl*II, R: *Eco*RI, Ms/Hp: isoschyzomers *Msp* I (methylation-insensitive) and *Hpa*II (methylation-sensitive), respectively. The location of the Jβ1 and Eμ 300 probes, as well as the size of the various fragments expected from specific cleavage, are indicated below the diagram. (**B**): thymus DNA from a transgenic mouse in the 382 (cVTECμ), 3−12 (cVTE), and 4−8 (cVTEμ800) lines and from a wild type mouse (−) was assayed using the Eμ 300 probe. BR: DNA restricted with *Bgl*II + *Eco*RI; Ms or Hp: (*Bgl*II + *Eco*RI)-restricted DNA further digested with *Msp* I or *Hpa*II, respectively. The size of the Eμ300-hybridizing fragments is indicated; Edg. refers to the germline (*Bgl*II−*Eco*RI: 2.4kb) Eμ-containing fragment from the IgH locus. Lanes 1−3 and 4−12 correspond to different experiments. (**C**): genomic DNA from G418-resistant clones of 38B9tk$^-$ cells co-transfected with substrates cVTECμ or cVTEμ800 was assayed using the Jβ1 probe. The size of the Jβ1-hybridizing fragments is indicated; Edg. refers to the germline (*Bgl*II−*Eco*RI: 4.3kb; *Bgl*II−*Msp* I: 0.7kb) Jβ1-containing fragments from the TCRβ locus.

results for three positive clones from each experiment are shown in figure 3. Significantly, all cVTEμ800, cVTE, and cVTECμ transfectants contain, in addition to the endogenous and unrearranged substrate fragments, roughly equivalent amounts of a submolar fragment that has the size predictive of DβJβ substrate rearrangements (lanes 1−6, 8−10). In contrast, the cVTCμ transfectants contain only the endogenous and unrearranged substrate fragments (lanes 11−13). Using PCR

assays, we have confirmed that substrate DβJβ joins are present in equivalent amounts in the cVTEμ800, cVTE, and cVTECμ transfectants (at ~ ten times lower levels than in thymus of the cVTECμ transgenic mice), but are absent in the cVTCμ transfectants (data not shown). Together, these results imply that the Eμ-containing fragment is necessary to induce rearrangements within recombination substrates transfected into pre-B cells, which is in agreement with the results from previous transgenic experiments (6). However, in marked contrast to what was observed in the lymphoid cells of the transgenic mice, Eμ-positive substrates can undergo similar level of DβJβ rearrangements in transfected pre-B cells, irrespective of the presence or absence of the downstream IgH sequences.

The changeable potentials for V(D)J recombination of the cVTE and cVTEμ800 substrates was reminiscent of the block in transgene expression occasionally observed following microinjection of certain DNA into mouse pre-implantation embryos as opposed to transfection into more differentiated cells (28). This selective inhibition of transgene expression has tentatively been attributed to the absence of natural introns or the presence of prokaryotic sequences in the microinjected insert (29−32). Whether either feature can also impair V(D)J recombination is not known. However, it should be noted that: i) significant amounts of intron/exon sequences were missing in the cVTE and cVTEμ800 constructs, as compared to the cVTECμ construct, and ii) consequently, in the cVTE and cVTEμ800 inserts, vector sequences were brought nearer to the Eμ regulatory region (see Fig. 1 and Methods). To assess the individual contribution of those sequences in our experimental system, we produced transgenic mice using an insert devoid of any sequences downstream of the 1.1kb Eμ-containing fragment (Fig. 4, left panel, top: VTE insert). Genomic DNA from thymus and kidney of fifteen mice born following VTE microinjections were digested with *Bgl*II and tested by Southern blot analysis, using the Jβ1 probe. When compared to a wild type mouse, the tissues of two animals (the 5.1 and 5.7 mice) contained additional Jβ1-hybridizing fragments which indicated that they carried the microinjected insert (Fig. 4, right panel). In addition, DNA from the thymus (but not the kidney) of both mice contains discrete fragments of sizes expected to carry substrate DβJβ and Vβ(Dβ)Jβ rearrangements (see figure legend for details on the different fragments labelled in the assay). As determined following densitometric scanning of hybridized filters, the relative intensities of these fragments and of the corresponding fragments found in mice carrying the cVTECμ substrate were in the same order of magnitude (within a factor of two difference; Fig. 4, left panel, bottom). Together, these results demonstrate that all IgH sequences located downstream of the 1.1kb Eμ-containing fragment (including the Eμ 3′ MARs) are dispensable for substrate rearrangement in thymus, although it should be stressed that our data do not rule out a role for MAR sequences in V(D)J recombination because the Eμ 5′ MARS are present in the VTE insert. Conversely, these results imply that the lack of V(D)J recombination within the cVTE transgenes was due to the vector sequences attached to the microinjected inserts. Because the same prokaryotic sequences did not avoid V(D)J recombination to occur at high frequency within the cVTECμ transgenes (6; this study), we conclude that the m region in the cVTECμ substrate somehow protects the transgenes against this suppressive effect. The suppression of recombination also observed within the cVTEμ800 transgenes indicates that the 820bp of Eμ 3′ flanking sequences are not sufficient for protection.

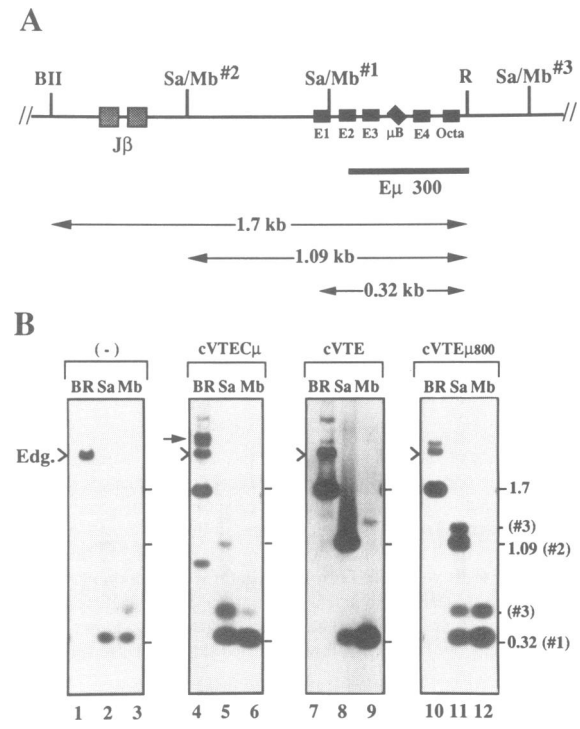

Figure 6. Methylation of dinucleotide CpG#3234 within the integrated substrates. (**A**): the strategy for the assay is depicted as in Figure 5; Sa/Mb: isoschyzomers *Sau*3AI (methylation-sensitive; only the *Sau* 3AI sites [sequence GATC] in which the cytosine residue is followed by a guanosine are susceptible of being altered by CpG methylation) and *Mbo*I (methylation-insensitive), respectively. Numbers indicate methylatable (#1) and non-methylatable (#2 and #3) *Sau*3AI sites on the restriction map. (**B**): thymus DNA from transgenic (lines 382, 3−12, and 4−8) or wild type (−) mice was assayed as outlined in (A); legends are as in Figure 5B. Sa or Mb: (*Bgl*II + *Eco*RI)-restricted DNA further digested with *Sau*3AI or *Mbo*I, respectively; #1 or #2 indicates *Sau*3AI (or *Mbo*I)/*Eco*RI fragments cut at site *Sau*3AI (or *Mbo*I) #1 or #2, respectively; additional upper fragments (#3) most likely correspond to *Sau*3AI (or *Mbo*I) fragments cut at *Sau*3AI (or *Mbo*I) site #3, after incomplete (partial) *Eco*RI cleavage. The arrow indicates Vβ(Dβ)Jβ substrate rearrangements in the cVTECμ transgenes.

Lack of substrate rearrangement correlates with hypermethylation of the transgenes

DNA modification by methylation profoundly affects both chromatin structure and gene expression (see 33,34 for reviews). Moreover, recent studies have indicated that DNA methylation can inhibit the V(D)J rearrangement potential of various types of recombination constructs (35,36). We therefore investigated the methylation status of our substrates in transgenic tissues or in transfected pre-B cells, focusing especially on the Eμ-containing region. In mammals, DNA methylation predominantly affects cytosine in CpG dinucleotides. The 1.1kb Eμ-containing fragment encompasses seven CpG motifs, of which two (positions #2435 and #3234 in locus MUSIGCDO7/GENBANK, accession# J00440) coincide with the sites of distinct methylation-sensitive endonucleases. CpG#2435 is placed within an *Hpa*II restriction site; at the IgH locus, this site becomes demethylated with the activation of the Eμ enhancer and the onset of B-cell differentiation (37). CpG#3234 is located at the border of a methylatable *Sau*3 AI restriction site that flanks the μE1 element; μE1 is one of the stimulatory elements within the Eμ enhancer known to bind nuclear factors in lymphoid cells (38,39).

Methylation of the two CpG dinucleotides in the recombination substrates was assessed by Southern blot analysis, using appropriate restriction endonuclease cleavage of genomic DNA and hybridization with specific probes (the strategy for these assays is depicted in Fig. 5A and 6A, respectively).

For each type of construct, analyses of independent lines of transgenic mice and of independent cell-transfectants gave consistent results. Representative results are shown in figures 5B/5C and 6B. In thymus, dinucleotide CpG$^{\#2435}$ was hypomethylated in the cVTECμ transgenes, whereas it was hypermethylated in the cVTE and cVTEμ800 transgenes, as shown by the sensitivity or resistance to cleavage of the relevant *Hpa*II restriction site (Fig. 5B, compare lanes 1−3, 7−9, 10−12); it is of note that the endogenous *Hpa*II site was resistant in thymus, a result in agreement with previous findings in murine T cell lines (37). Using similar assays, hypomethylation at the same site within the transgenes was observed in thymus of the 5.1 mouse carrying the VTE insert, but not in those of mice carrying the cVTCμ insert (data not shown). In contrast, in pre-B cell-transfectants, a roughly equivalent profile of CpG methylation was observed among the various Eμ-containing substrates. Thus, in both the cVTECμ or cVTEμ800 substrates, the *Hpa*II site which encompasses CpG$^{\#2435}$ was relatively resistant to cleavage (Fig. 5C), implying the overall methylation of this sequence in the 38B9 transfectants. Interestingly however, submolar amounts of the *Bgl*II−*Hpa*II (0.6kb) Jβ-hybridizing fragment observed in DNA from the cVTECμ and cVTEμ800 transfectants (lanes 3 and 6) were not detected using the enhancerless cVTCμ transfectants (not shown) suggesting that a low level of CpG$^{\#2435}$ hypomethylation prevails within the Eμ-containing substrates. Analysis of dinucleotide CpG$^{\#3234}$ using *Sau*3 AI-digested thymic DNA also yielded consistent results. For example, the relative accessibility of cleavage of the *Sau*3 AI site flanking the mE1 element indicated that CpG$^{\#3234}$ was hypomethylated in the cVTECμ and VTE transgenes and, conversely, more methylated in the cVTE and cVTEμ800 transgenes (Fig. 6B, lanes 4−6, 7−9, 10−12, and data not shown). Note that the endogenous site was accessible to *Sau*3 AI-cleavage in the thymus (see for example lanes 1−3), indicating that CpG$^{\#3234}$, in contrast to CpG$^{\#2435}$, is prone to hypomethylation in this organ (however, both dinucleotides in the transgene and in the endogenous IgH gene were hypermethylated in kidneys—data not shown). In summary, an increased level of CpG methylation within the Eμ-containing fragment is specifically observed in the cVTE and cVTEμ800 versus the cVTECμ and VTE transgenes. Further analyses suggested that in thymus the 5′ Dβ region of the cVTE and cVTEμ800 transgenes was also hypermethylated when compared to that of the cVTECμ transgenes (C.Fernex, unpublished data). Together, these results are best explained assuming that the presence of the prokaryotic sequences in the vicinity to the Eμ-regulatory elements (and/or the upstream variable gene segments) induces a de novo methylation of the flanking regions within the microinjected substrates which somehow leads to a block of V(D)J rearrangement, although a direct effect of the prokaryotic sequences on V(D)J rearrangement, followed by incidental methylation of the transgenes, cannot formally be ruled out (see also below). Because methylation patterns are essentially established in a series of dynamic changes over the course of gametogenesis and early embryogenesis (40), it is conceivable that important variations in the level of CpG methylation of the various inserts, and therefore in the potential of V(D)J recombination, may occur when they are introduced into pre-implantation embryos. The significant level of CpG methylation observed among the various recombination substrates introduced into more differenciated cells, such as the 38B9 pre-B cells, may reflect the tendency of cultured cell-lines to progressivly methylate nonessential genes (41), rather than a prokayotic-induced effect.

A variety of transgenes have been described which display lack of expression and concomitant CpG hypermethylation. In several instances however, only one or few transgenic lines carrying the same construct were affected, suggesting that the site of integration, and not the transgene itself, was the target for the observed modifications (see 42,43 for examples). In contrast, our hypothesis that the prokaryotic sequences target the epigenetic changes and the suppressive effect on V(D)J recombination is reinforced by the findings of hypermethylation and coincidental lack of rearrangements within two related (i.e. cVTE and cVTEμ800) substrates in several independent transgenic mouse lines. In this regard, our findings parallel the behaviour of another recombination substrate, the pHRD construct (44,45). This construct was hypermethylated and concurrently inert for V(D)J recombination in all independently derived transgenic mouse lines, whereas it was competent for rearrangement when transfected into pre-B cells. The mechanism of hypermethylation was apparently mediated through a single strain-specific modifier (Ssm-1) active in a C57BL/6 strain background. Likewise, the pHRD construct carries sequences of prokaryotic origin near its variable-gene-segment and Eμ-enhancer components. Taking the view that methylation may constitute a system for neutralizing invading foreign DNA (46), it is tempting to speculate that our transgenes may also be controlled by the Ssm-1 (the transgenic lines were maintained by crossing with [C57BL/6×CBA] F1 mice) or related modifier(s) directly involved in such a neutralizing mechanism. The generality of this phenomenon is further suggested by the suppressive effect on immunoglobulin light chain kappa (IgLκ) rearrangements, following insertion of the bacterial Neo gene downstream of the IgLκ intron enhancer (47).

Evidence has been presented which implies that methyl CpGs may either directly interfere with the binding of specific trans-activating factors or, alternatively, be bound by nuclear proteins which indirectly induce an inaccessible DNA conformation (33,34). Conceivably, either direct and/or indirect mechanisms can account for the inhibition of V(D)J recombination that we observed by affecting the access to their target sequences of critical diffusable factor(s), such as V(D)J recombinase component(s) or Eμ trans-activator(s), and/or by affecting putative interactions between such factors. The suppressive effect might result from the spreading of the prokaryotic-induced methylation into the flanking eukaryotic sequences within the transgenic substrates, a model that bears similarities with variegating positional effects (48). In the cVTECμ transgenes, the spreading could then be limited either passively by the length (about 10kb) of the IgH Cμ-containing region or actively by some ill-defined element(s) present in this region (49,50). Structural features in DNA of non-vertebrate origin, such as the content of CpG, may contribute further to the suppressive effect. In fact, the first 100bp of pMCS vector sequences attached to the 3′ side of our constructs—a part of the Tn3 transposon of *E.coli*—include 8 CpGs which is higher than the average 1/100 ratio characteristic of the mammalian genome (51). Moreover, they also contain a sequence homologous to the murine R repetitive element. Interestingly, sequences homologous to a related (B1) repetitive

element have been described in negatively acting elements at the Igκ and TCRα loci (52).

Recently, gene targeting experiments have been used to investigate Ig transcriptional and/or recombinational cis-acting elements. Most of the experimental approaches resulted in the replacement of the regulatory element(s) in the murine genome by a selectable gene or by recombination target sequences of non-vertebrate origins (14,47,53–55). Our data point out that the mere insertion of such sequences may not be neutral with respect to the chromatin structure and recombinational accessibility of the targeted region. This may explain, at least in part, the quantitative differences seen when analyzing the consequences on V(D)J recombination of targeted deletion versus targeted replacement of enhancer-containing DNA fragments (13,14,47).

ACKNOWLEDGEMENTS

We would like to thank Drs F.Watrin, G.Ku, and K.Crook for critically reading this manuscript. This work was supported by grants from the 'Institut de la Santé et de la Recherche Médicale', the 'Centre National de la Recherche Scientifique', and the 'Association pour la Recherche sur le Cancer'.

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