Immunogenetics 37: 331–336, 1993

Immuno-genetics

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Original articles

The mouse *Eb* meiotic recombination hotspot contains a tissue-specific transcriptional enhancer

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Received June 3, 1992; revised version received August 13, 1992

Abstract. A meiotic recombination hotspot exists within the second intron of the mouse major histocompatibility complex (MHC) gene, *Eb*. In the present study, a small fragment from the intron which contains two potential transcriptional regulatory elements was cloned into an expression vector and its effect on transcription was tested. This fragment was found to contain tissue-specific transcriptional enhancer activity. An octamer-like sequence and a B motif may contribute to this enhancer activity. Similar regulatory sequences with the same orientation and distance from one another are found in another mouse MHC recombination hotspot.

Introduction

The second intron of the mouse class II major histocompatibility complex (MHC) gene, *Eb*, contains a meiotic recombination hotspot (Begovich and Jones 1985; Kobori et al. 1986; Saha and Cullen 1986; Steinmetz et al. 1982). Recently, a major DNase I hypersensitive site was found in this intron in meiotic cells isolated from mouse testes (Shenkar et al. 1991). Analysis of the sequences adjacent to this site revealed a segment containing an octamer-like sequence, and a B motif that is potentially able to bind H2TF1/KBF1 and NFkB transcription factors. Gel retardation experiments showed specific protein binding to each of these two potential binding sites (Shenkar et al. 1991). In the present study, we assayed the enhancer function and tissue specificity of this fragment from the recombination hotspot region.

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Materials and methods

Construction of plasmids. An *Eb* fragment containing the potential binding sites was obtained by polymerase chain reaction (PCR) amplification of 2PK-3 cell genomic DNA with two primers; 5′-tccaAGCTTCTTCACTCAGCTTCAG-3′ and 5′-GTGCTGCAGTCTGCACCCTGACC-3′. The sequences in capital letters are complementary to *Eb*, while the nucleotides in lower case were added to the 5′ end of the first primer to introduce a *Hin* dIII restriction enzyme site. The 5′ end of the second primer contained a naturally occurring *Pst* I site. Treatment of the PCR product with these two enzymes produced a 269 base pair (bp) fragment (Fig. 1) that contains sequences extending from position 1514 to 1782 of the *Eb* gene (Kobori et al. 1986) and was used for plasmid construction. The plasmid pUC-OT [Fig. 2; also called OTCO by DeFranco and Yamamoto (1986)] was used as a vector to construct plasmids containing the *Eb* fragment and as a control plasmid in the enhancer activity assay. pUC-Eb(*) was constructed (Fig. 2) by cloning the 269 bp *Hin* dIII-*Pst* I *Eb* fragment into the *Hin* dIII and *Pst* I sites of pUC-OT. All but the 3′ most 42 bp of the tk-promoter fragment was removed in this procedure. pUC-Eb(*) was used to test for any possible promoter activity of the insert itself. pUC-Eb(+) was made (Fig. 2) by ligating *Hin* dIII and *Bam* HI-digested pUC-OT with an *Eb* fragment obtained by cleaving pUC-Eb(*) with *Hin* dIII and *Bgl* II. These latter sites are located uniquely in the polylinker and plasmid sequence, respectively. The insert in plasmid pUC-Eb(–) was obtained (Fig. 2) by digesting pUC-Eb(*) with *Pst* I, trimming with T4 DNA polymerase to produce blunt ends, followed by cutting with *Hin* dIII. The *Eb* insert carried a *Hin* dIII and blunt end and was cloned into the vector, pUC-OT, after the latter was cleaved with *Nar* I, blunt ended by filling in with Klenow, followed by *Hin* dIII digestion. Plasmid p65VALO [described as plasmid Z by Picard and Yamamoto (1987)] containing a *Lac-Z* gene was used as an internal control for transfection and the chloramphenicol acetyltransferase (CAT) assay. All these plasmids were purified by CsCl gradient centrifugation.

Cell culture, transfection, and cell extraction. The mouse B-cell lymphoma line, 2PK-3 (ATCC-TIB 203, $H\text{-}2^d$), was maintained in Dubelco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), 1% L-glutamine, 0.05 mM 2-mercaptoethanol and 1% streptomycin-penicillin. 2PK-3 cells (2.5×10^7 cells in 0.5 ml of serum-free DMEM medium) were transfected by electroporation [960 μF and 220 V; BioRad Gene Pulser (BioRad, Richmond, CA)]

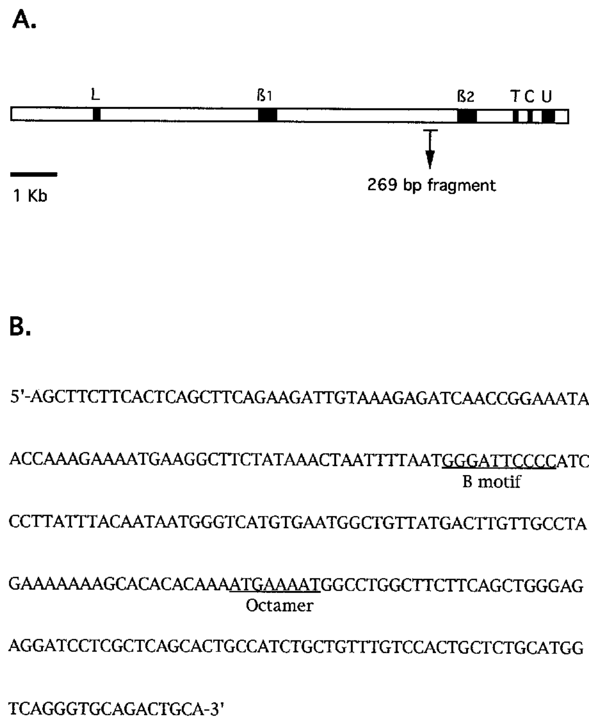

Fig. 1 A, B. Location and sequence of the *Eb* fragment in the mouse *Eb* gene. L, β_1, β_2, C, T, and U refer to the leader, beta-1, beta-2, cytoplasmic, transmembrane, and 3′ untranslated exons. The DNA sequence of the 269 bp *Eb* fragment in the second intron is shown *below* and extends (5′ to 3′) from position 1514 to 1782 according to the numbering system of Kobori and co-workers (1986).

using 21 µg of the test plasmid and 18 µg of the control plasmid P65VALO. Sixty-six h after electroporation, a portion of the cell extract was prepared for the β-galatosidase assay. The remainder of the extract was prepared for CAT activity assays. The mouse connective tissue cell line (L-M, ATCC-CCL 1.3, $H2^k$, derived from a C3H/An strain) was grown in DMEM containing 10% FBS and 1% streptomycin-penicillin. Transfection of this cell line was carried out using lipofectin (Felgner et al. 1987). Cells grown to 80–90% confluence were washed in serum-free DMEM. The cells were incubated with 8.0 µg of test plasmid, 5.0 µg of p65VALO and 30 µg of lipofectin (BRL Laboratories, Gaithersburg, MD) in serum-free DMEM for 10 h at 37° C, 7% CO_2 followed by incubation in serum-containing DMEM for 60 h. Cell extracts were made as described above.

Enzyme assays. The protein concentration of each extract was measured using the BioRad Protein Assay. β-galatosidase activities were determined by the method of DeFranco and Yamamoto (1986). CAT activities were measured by the TLC method of Gorman and co-workers (1982), and quantitated by cutting out the products from the TLC plate followed by liquid scintillation counting.

Analysis of Eb mRNA. L-M or 2PK-3 cells (*H-2*^{k} and *H-2*^{d}, respectively) were washed twice with isotonic Tris buffer (pH 8.0) containing 1 mM ethylenediaminetetraacetate (EDTA) and counted. Ten-thousand cells were centrifuged, the buffer removed, 14 µl of water added and the tube placed in a boiling water bath for 10 min followed by centrifugation to remove any debris. The whole sample was reverse transcribed according to the procedure of Kawasaki (1990) using 0.5 µM of the primer (5′-AGCAGACCAGGAGGTTGTGG-3′) which is complementary to a portion of the third exon (*Eb*-β*2*) of the *Eb* gene. After a 10 min, 95° C denaturation step, 50 cycles of PCR were carried out (95° C denaturation for 1 min, 60° C annealing for 1 min, 72° C extension for 5 min) using this same primer and a second primer (5′-ACGCCGAGAACTGGAACAGC-3′) from within the second exon (*Eb*-β*1*). These two primers are identical with the *Eb*^{d} and *Eb*^{k} second exon sequences (Wildera and Flavell 1984) and will amplify cDNA from both haplotypes. The primers were used at 1 µM each. To detect amplified *Eb* cDNA, dotblot hybridization (Saiki et al. 1986) was used with a ^{32}P labeled probe (5′-GCGGAGAGTTGAGCCTACGG-3′). The probe was complementary to the last 8 bp of the *Eb*-β*1* exon and the first 12 bp of the *Eb*-β*2* exon. This probe could only hybridize to PCR product from spliced *Eb* message.

Results

We first determined the steady state level of *Eb* mRNA in 2PK-3 lymphoma cells and L-M fibroblast cells. Whole cells were lysed and subjected to reverse transcription with an *Eb* specific primer from the *Eb*-β*2* exon. PCR product made using this primer and one from the upstream *Eb*-β*1* exon was spotted on a nylon filter and hybridized to a probe specific for cDNA that was derived from RNA which had undergone splicing between the two exons. The results (Fig. 3) show that

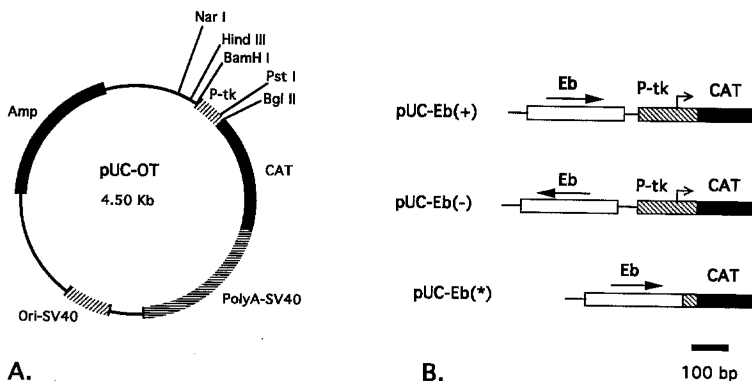

Fig. 2 A, B. Structure of plasmids used to test the *Eb* fragment for enhancer activity. **A** Plasmid vector showing the location of important restriction enzyme sites and functional regions. **B** Organization of the three different plasmids containing the *Eb* fragment in the region adjacent to the *CAT* gene. A *bar* representing a 100 bp segment of DNA is also shown. P-tk: thymidine kinase promotor. Plasmid construction details are as discussed in Materials and methods.

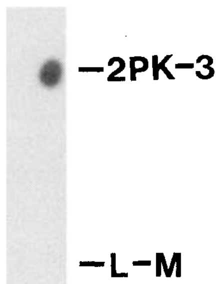

Fig. 3. Steady state levels of *Eb* mRNA in 2PK-3 and L-M cells. Even after an autoradiographic exposure 24 times that shown, no signal could be detected in L-M cells.

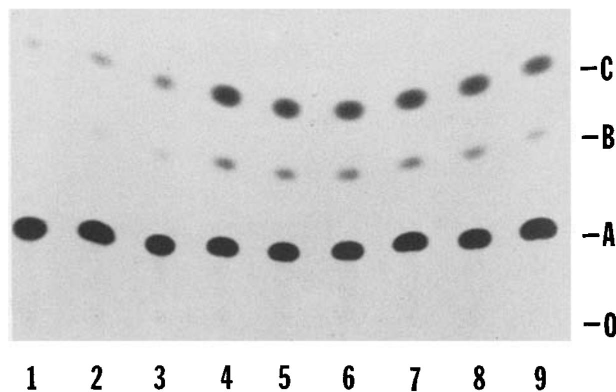

Fig. 4 A – C. Results of analysis of enhancer activity in the *Eb* fragment. **A** CAT assay results from three replicates using 2PK-3 cells transfected with pUC-OT (lanes 1–3): pUC-Eb(+), lanes 4–6; and pUC-Eb(–), lanes 7–9. O: origin of chromatography. **A** ^{14}C chloramphenicol; **B** and **C** Show the two different acetylated reaction products.

Eb mRNA is abundant in 2PK-3 cells but cannot be detected in L-M fibroblast cells.

To determine the effect of the *Eb* fragment on transcription, pUC-OT, pUC-Eb(+), pUC-Eb(–), and pUC-Eb(*) were each individually transfected along with the β-galactosidase control plasmid p65VALO, into 2PK-3 or L-M cells. The tissue-specific transcriptional enhancer activity of the *Eb* segment is judged by comparing the CAT assay results from the 2PK-3 and L-M cell lines. The addition of cell extracts from untransfected cells or no extract at all gave no product after thin-layer chromatography. The data for 2PK-3 cells (Fig. 4; Table 1), based on four replicates, show that the *Eb* fragment enhances CAT gene expression approximately fourfold in both orientations [pUC-Eb(+) and pUC-Eb(–)] compared with the control plasmid without the *Eb* fragment (pUC-OT). The tk-promoter-deleted plasmid, which contains the *Eb* fragment [pUC-Eb(*)], has little if any CAT gene expression. It can be concluded that the *Eb* fragment contains orientation-independent transcriptional enhancer activity without any promoter activity.

In contrast, we did not observe any enhanced CAT gene expression when the *Eb* fragment-containing plasmids were transfected into L-M cells (Fig. 5; Table 1). The CAT activities for pUC-Eb(+) and pUC-Eb(–) are even slightly lower than that for the pUC-OT control plasmid. This result indicates that the *Eb* fragment acts as an enhancer in a tissue-specific manner.

Discussion

Studies on several class II genes, both mouse and human, have revealed a number of transcriptional regulatory sequences upstream and downstream of the transcriptional initiation site. The upstream sequences have been proven to contain the main tissue-specific promoter and enhancer activities (Dorn et al. 1987; Sherman et al. 1989a, b; Sakurai and Strominger 1988; Koch et al. 1988, 1989; Saito et al. 1983; Finn et al. 1990a, b). Downstream transcriptional enhancer activity has been identified in human *DRA*, *DQA*, and *DQB* genes (Benoist and Mathis 1990; Wang et al. 1987; Sullivan and Peterlin 1987) and now in mouse *Eb*. The actual roles if any, that any of these downstream regions play in class II gene expression in vivo is unknown.

Two regulatory sequences could account for the enhancer activity of the *Eb* fragment in 2PK-3 cells. One is an inverted octamer sequence, the other a B motif

Table 1. Transcriptional enhancer analysis of the *Eb* DNA fragment in 2PK-3 and L-M cells.

Plasmids	2PK-3 cells			L-M cells		
	n	$\bar{X}$±S. E	R	n	$\bar{X}$±S. E	R
pUC-OT	4	7,268± 664	1.00	4	29,282±1594	1.00
pUC-Eb(+)	4	32,031±1728	4.41	4	25,523±3579	0.87
pUC-Eb(–)	4	29,104±2435	4.00	4	21,698±1472	0.74
pUC-Eb(*)	1	76	0.01		N/A	

CAT activities are expressed in counts per min of the pooled acetylated reaction products (**B** and **C**; Fig. 4). For the two cell lines, CAT activity for each plasmid is normalized to the activity of the pUC-OT vector. Raw data from the two different cell lines cannot be compared directly because of different transfection efficiencies and different amounts of cell extract used in the CAT assay. n: number of replicates; X: mean number of cpm for the four replicates; S. E: standard error of the mean; R: ratio of CAT activities normalized to pUC-OT.

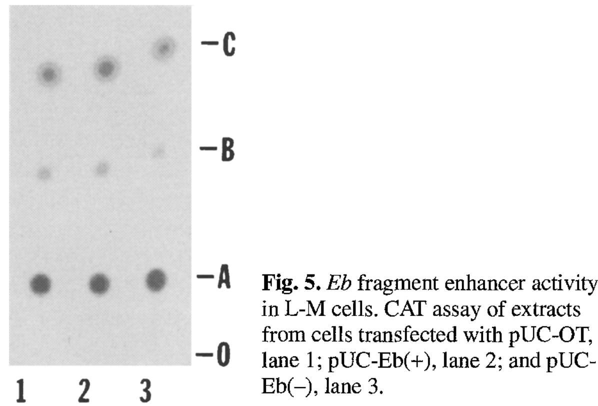

Fig. 5. *Eb* fragment enhancer activity in L-M cells. CAT assay of extracts from cells transfected with pUC-OT, lane 1; pUC-Eb(+), lane 2; and pUC-Eb(–), lane 3.

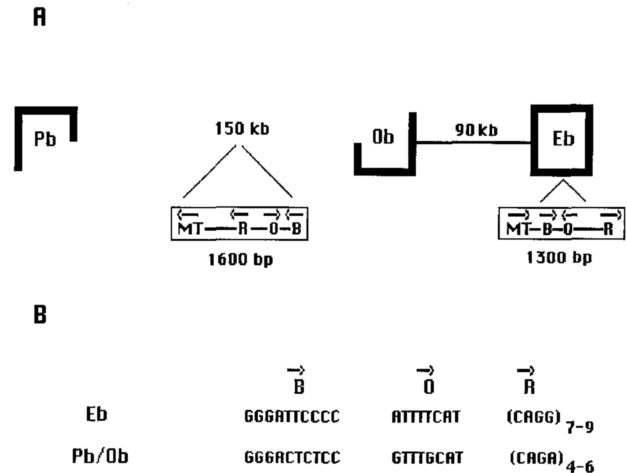

Fig. 6. **A** Comparison of structures which overlap mouse *Eb* and *Pb/Ob* recombination hotspots. *Eb*, *Pb*, and *Ob* genes are each *heavily outlined*. The two *lightly outlined rectangles* include the transcription factor binding motifs (*O* = octa, B = B motif); MT: mouse MT repeated sequence family; R: tetranucleotide repeats. *Arrows* denote orientation of these sequences. **B** Similarity among the B, O, and R sequences in the two hotspots. At *Eb*, the B sequence is found between positions 1601 and 1610, the O sequence between 1684 and 1691, and the repeats begin at 2217 according to the numbering system of Kabori and co-workers (1986). In the hotspot between *Pb* and *Ob*, the B sequence is found between positions 1550 and 1560, the O sequence between 1465 and 1472, and the repeats begin at 1135 according to the numbering system of Shiroishi and co-workers (1990).

which is the binding site for a number of transcription factors, e. g., H2TF1/KBF1 and NFkB. These two regulatory elements are important for B-cell-specific transcription of several immunoglobulin (*Ig*) genes and class II MHC genes (Sherman et al. 1989b; Pierce et al. 1988; Lefranc and Lefranc 1990). Thirty bp oligonucleotides, each containing one of these two elements, were previously shown to be bound by proteins in a 2PK-3 nuclear extract in gel-retardation experiments (Shenkar et al. 1991).

The octamer motif is a key control element for B-cell-specific gene expression of *Ig* and *HLA-DRA* (Sherman et al. 1989b), and also for some non-B-cell-specific gene expression (Kemler et al. 1989, 1991; Lefranc and Lefranc 1990). The inverted octamer sequence of the *Eb* fragment (ATTTTCAT) is similar to the consensus octamer motif (ATTTGCAT) with one base substituted, but is identical to the octamer sequence found in the intronic enhancer region of the mouse T-cell receptor β-chain gene (Krimpenfort et al. 1988) and the intronic enhancer of the human *IgH* gene region (Maeda et al. 1987).

The B motif also functions in the control of B-cell-specific and non-B-cell-specific transcription, and binds several different trans-acting factors (Lenardo and Baltimore 1989). The binding protein, NFkB, is responsible for transcriptional regulation of a variety of genes involved in the immune response (Lenardo and Baltimore 1989). Another B motif-binding protein, H2TF1, increases the transcription of the mouse $H2k^b$ class I gene and is found in most cell types (Baldwin and Sharp 1988).

The B motif in the *Eb* fragment (GGGATTCCCC) is more similar to the H2TF1 binding site (GGGGATTCCCC) in the $H\text{-}2K^b$ promoter (Baldwin and Sharp 1987) than the one found in the mouse kappa light chain gene (GGGACTTTCC; Sen and Baltimore 1986). However, the B motif in the *Eb* fragment contains GGGA at its 5′ end and CC at its 3′ end which are conserved in sequence in B motif variants (Zabel et al. 1991).

The *Eb* meiotic recombination hotspot in the second intron has been defined quite precisely by the study of many recombinant mouse strains, reviewed by Bryda and co-workers (1992). Another major meiotic recombination hotspot (Uematsu et al. 1986; Shiroishi et al. 1990, 1991) exists in the mouse *H-2* region adjacent to the *LMP2* gene (Martinez and Monaco 1991) within the interval bounded by the *Pb* and *Ob* genes.

Figure 6A summarizes some of the DNA sequence features which have been proposed as playing a role in both the *Eb* and *Pb/Ob* recombination hotspots. These elements include the members of the mouse MT repeated sequence family which has been suggested as playing an especially important role in recombination (Shiroishi et al. 1990; Bryda et al. 1992) and tetranucleotide repeats (Kobori et al. 1986). Both kinds of sequences are contained within a 1300–1600 bp DNA segment which overlaps the two recombination hotspots. We previously localized the octamer and B motifs to the *Eb* intron after inspection of the DNA sequences adjacent to a DNase I hypersensitive site we found in mouse cells undergoing meiosis (Shenkar et al. 1991). Inspection of the DNA sequence in the *Pb/Ob* hotspot region also revealed an octamerlike and B-motif-like sequences separated by almost exactly the same distance as in the *Eb* hotspot (70–80 bp). The orientation of these motifs and the sequence similarity

between them in the two hotspot regions is shown in Figure 6B. Whether the motifs adjacent to *LMP2* play a role in the transcription of this gene remains to be established.

Mitotic recombination hotspots in yeast have been associated with increased transcription, and some recombination hotspots are associated with active promoters (Thomas and Rothstein 1989; Voelkel-Meiman et al. 1987). Intrachromosomal somatic recombination mediated by VDJ recombinase during lymphocyte differentiation is also stimulated by transcription (Blackwell et al. 1986; Blackwell and Alt 1989). However, transcription does not appear to be a prerequisite for all recombination hotspot activity as demonstrated by a recent study on a hotspot active in yeast meiosis (Nicolas et al. 1989; Schultes and Szostak 1990). If recombination enhancement results from an open chromatin structure (Blackwell et al. 1986), then the act of transcription itself may not be required. The interaction of any one particular transcription factor with DNA may be a prerequisite but may not be sufficient for transcription. However, the binding of a transcription factor to DNA could introduce changes in chromatin structure (Gross and Garrard 1988) leading to enhanced accessibility of the DNA to the cells' recombination machinery (Shenkar et al. 1991). Recent data consistent with this hypothesis comes from studies on the fission yeast *Schizosaccharomyces pombe*. The sequence ATGACGT is required for recombination hotspot activity in the *ade 6* gene of this organism, and a protein which binds this sequence has been identified in extracts from meiotic cells (Schuchert et al. 1991; Ponticelli and Smith 1992). If the binding of transcription factors contributes to the origin of recombination hotspots, then recombination might occur at higher frequencies in chromosomal segments rich in expressed genes. It is of interest to note that, based upon a comparison of genetic maps in eucaryotic species with both large and small genomes, Thuriaux (1977) proposed that there might be a tendency for recombination to occur preferentially in structural gene regions.

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