Cell type-specific enhancer element associated with a mouse MHC gene, E_β

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Class II molecules of the major histocompatibility complex (MHC) are heterodimeric glycoproteins expressed on the surface of antigen-presenting B lymphocytes and macrophages[1]. The genes encoding the α- and β-chains of the class II heterodimers, $A_\alpha A_\beta$ and $E_\alpha E_\beta$, have recently been characterized at the molecular level[2-5], and certain cloned genes were shown to be functionally expressed after introduction into cells by DNA-mediated gene transfer[6,7]. One study[7] found that a transfected E_β^b gene was expressed in a macrophage cell only after treatment of cells with γ-interferon. DNA sequences associated with transfected Class II MHC genes may therefore have a regulatory role in their cell type-specific expression. We report here the identification of a cell type-specific transcriptional enhancer element associated with the mouse E_β^d gene.

We[8] and others[9,10] have recently shown that the tissue-specific expression of immunoglobulin heavy-chain genes is regulated by an immunoglobulin heavy-chain gene-associated enhancer element located between J_H and C_μ. This enhancer functions in cells which normally express heavy-chain genes but not in cells derived from other tissues, for example, fibroblasts. The possibility that an enhancer element might be associated with the mouse E_β gene was tested by subcloning restriction fragments of the gene and its flanking regions into the EcoRI site of plasmid pSER and testing the ability of the recombinant plasmids to transform the B lymphoma line, A20-2J (ref. 11) to the gpt$^+$ phenotype (growth in the presence of mycophenolic acid). This plasmid was derived from plasmid pSV2gpt (ref. 12) by removing the simian virus 40 (SV40) enhancer sequence[8]. In the absence of any E_β gene fragments, plasmid pSER transformed A20-2J cells to gpt$^+$ at a frequency of $<5 \times 10^{-6}$ while the transformation frequency obtained with the parental plasmid, pSV2gpt (containing the SV40 enhancer) was $\sim 10^{-4}$. We conclude from these data and similar results with the myeloma cell line, J558L (ref. 8), that plasmid pSER is dependent on the addition of enhancer sequences for efficient transformation.

Only one region out of 28 kilobases (kb) of E_β coding and flanking sequences (Fig. 1) was found to be positive in this assay—a 4.1-kb HindIII–EcoRI fragment containing the first exon and approximately 2.7 kb of upstream sequence (see Table 1a). As the site of insertion of this fragment in plasmid pSER is some distance (2.5 kb) away from the SV40 promoter, and the 4.1-kb fragment worked equally well in both orientations (see below), we tentatively conclude that an enhancer element is located in this region of the E_β gene.

The subfragments shown in Fig. 1 were then tested for their ability to substitute for the SV40 enhancer. These fragments included three which extend from the 5' end of the 4.1-kb fragment (HindIII site) but differ at their 3' ends. As seen in Table 1b, fragments extending either to the BstXI site near the E_β gene promoter (HX fragment) or to the BamHI site at about −600 base pairs (bp) (HB fragment) were shown to contain full enhancing activity. The BstXI to EcoRI fragment (XR), containing the first E_β gene exon and a portion of the first intron, had no detectable activity. We have previously reported that two short sequences present upstream of the E_β gene promoter are conserved in the corresponding regions of the murine E_α and DR_α genes[5] (see Fig. 3). As the HB and HX fragments worked equally well in the enhancer assay, even though these sequences were not contained in the HB fragment, we conclude that these elements are not necessary for enhancing activity.

Subfragments of the 2.0-kb HB fragment were negative in the pSER assay (Table 1). These include the fragments produced by cleavage with PvuII (HP and PB) and a fragment spanning the PvuII site (AA). Thus it seems that the sequences required for enhancer activity are scattered over the 2.0-kb HB fragment.

Next we tested the recombinant plasmids that gave positive results in A20-2J cells for enhancing activity in other murine cell types (Table 1). No activity was detected when the 4.1-kb or HB fragment recombinants were used to transfect fibroblasts (L cells), indicating that this enhancing activity is tissue-specific. These same plasmid constructs also gave negative results in a myeloma J558L, a tumour of plasma cells which are terminally differentiated B-lineage cells not expressing class II antigens. In contrast, the pSER recombinant containing the immunoglobulin heavy-chain enhancer[8] was positive in both the B-lymphoma (A20-2J) and myeloma lines. We conclude that the cell type-specific expression of the E_β gene is due, at least in part, to the functionality of its associated enhancer element.
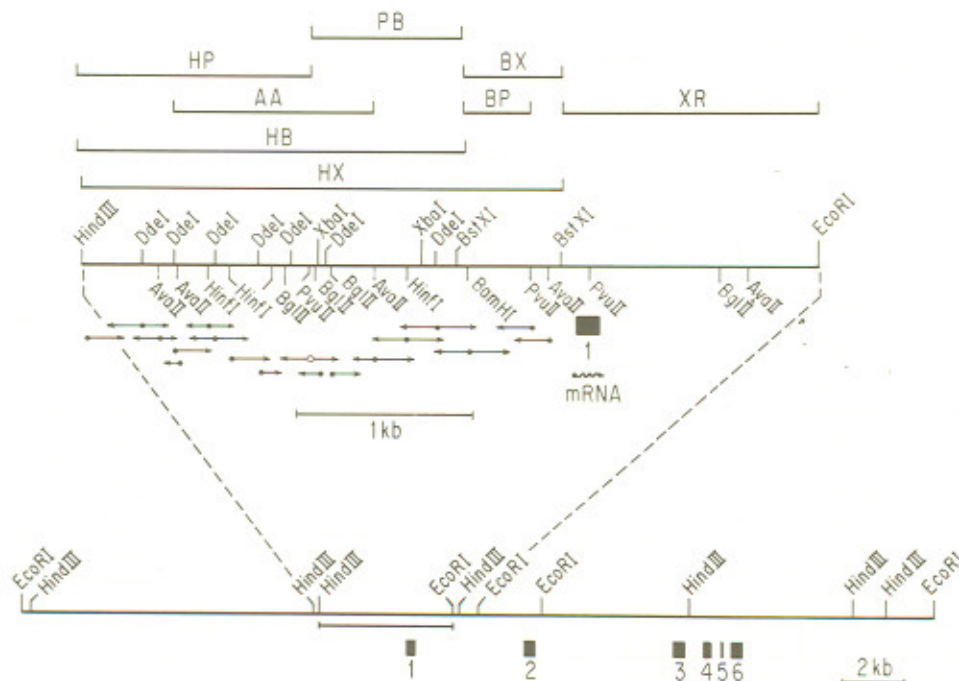
Fig. 1 Restriction map of the mouse E_β^d gene and flanking regions. The locations of the coding exons (solid boxes) are from ref. 5. The direction of transcription is from left to right. The 4.1-kb HindIII to EcoRI site is enlarged and the sequencing strategy of the 5'flanking region is indicated with arrows. Subfragments used to test enhancing activity (Table 1) are shown above and are abbreviated by letters which correspond to their 5' and 3' restriction sites.
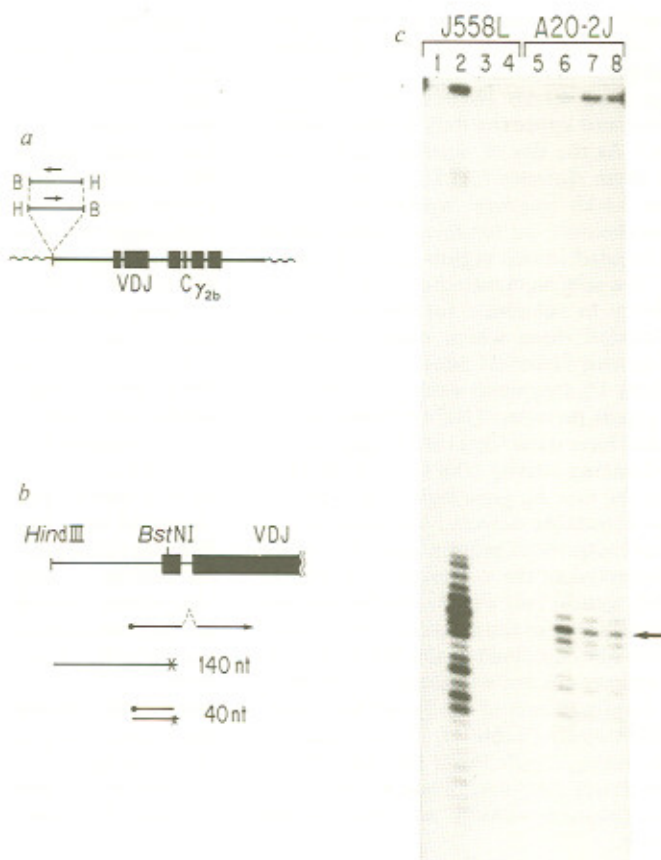
Fig. 2 Enhancement of γ2b heavy-chain mRNA transcription in J558L myeloma and A20-2J lymphoma cells. a, Diagram of plasmid pSV-γ2bΔX$_{2/4}$ (ref. 8) and the site of insertion of the 2.0-kb HB fragment from the 5′ flanking region of the E_β^d gene. This plasmid contains a functionally rearranged γ2b heavy-chain immunoglobulin gene but the heavy-chain enhancer has been removed (see Gillies et al.,[8] for details). The HB fragment was inserted in both orientations ~1.4 kb upstream of the γ2b promoter. b, Diagram of the S$_1$ nuclease protection assay used to measure γ2b mRNA levels. A single-stranded HindIII to BstNI fragment (140 nucleotides, nt) was hybridized to total cell RNA (20 μg) from transfected and control cells. The 40-nucleotide major protected fragment is indicated. c, Electrophoretic analysis of fragments protected by RNA from cells transfected with plasmid pSV-γ2bΔX$_{2/4}$ (lanes 1 and 5) and recombinants containing the heavy-chain enhancer (lanes 2 and 6) or the E_β^d HB fragment in the normal (lanes 3 and 7) or reversed orientations (lanes 4 and 8). An arrow indicates the 40-nucleotide major protected fragment.

γ2b mRNA could be detected in J558L cells transfected with the constructs containing the E_β HB fragment in either orientation (lanes 3 and 4), even with much longer exposures of the autoradiogram (not shown). A lower level of γ2b mRNA (relative to J558L cells) was detected in A20-2J cells transfected with the plasmid construct containing the heavy-chain enhancer fragment (lane 6). In contrast to the results with J558L cells, A20-2J cells transfected with the E_β HB fragment constructs contained levels of γ2b mRNA that were comparable to that seen in the same host cell (A20-2J) with the heavy-chain enhancer (lanes 7 and 8).

Transfected cell lines were analysed further by Southern blotting to determine the plasmid copy numbers (data not shown). Except for the J558L line, obtained by transfection with the heavy-chain enhancer-containing plasmid (one or two copies per cell), all of the lines of both A20-2J and J558L contained a high copy number (about 40 copies per cell). This dramatic effect of the heavy-chain enhancer on the plasmid copy number in J558L transfectants, obtained by gpt selection, was described previously[8]. When the level of γ2b mRNA (determined by S$_1$ nuclease protection; Fig. 2) is normalized to the number of gene

Table 1 Relative transformation frequencies of pSV2gpt and derivative plasmids

		Transfected cells		
	Plasmid	A20-2J	J558L	L
a				
	pSV2gpt	1.0		
	pSER	<0.1		
	pSER−13+pSER−13 R	0.55		
	pSER−0.7+pSER−0.7 R	<0.1		
	pSER−1.9+pSER−1.9 R	<0.1		
	pSER−12+pSER−12 R	<0.1		
	pSER−8.6+pSER−8.6 R	0.05		
	pSER−4.1+pSER−4.1 R	1.1		
b				
	pSV2gpt	1.0	1.0	1.0
	pSER	<0.05	0.02	0.05
	pSER−X$_{2/3}$ (heavy chain)	1.9	1.5	0.05
	pSER−4.1 R	1.6	<0.02	0.04
	pSER−4.1	1.6		
	pSER−HX R	1.9		
	pSER−HX	1.9		
	pSER−BX R	0.17		
	pSER−BP R	<0.05		
	pSER−HB R	2.0	<0.02	0.05
	pSER−HB	2.0		
	pSER−PB R	<0.05		
	pSER−HP R	<0.05		
	pSER−AA R	<0.05		
	pSER−XR R	<0.05		

Cells were transfected by protoplast fusion as described[8] and plated at 2×10^3 cells per well (A20-2J), 10^4 cells per well (J558L) or 10^4 and 10^5 cells per 100 mm dish (L cells). Selective medium contained mycophenolic acid at 1 μg ml^{-1} (A20-2J), 6 μg ml^1 (J558L) or 25 μg ml^{-1} (L cells). The transformation frequencies were normalized to a value of 1.0 which was assigned to the frequency obtained with plasmid pSV2gpt (10^{-4} for A20-2J, 4×10^{-4} for J558L and 2×10^{-3} for L cells). a, Four EcoRI fragments (13, 0.7, 1.9 and 12 kb long) covering ~28 kb of the E_β gene and its flanking regions (see Fig. 1) were cloned in both orientations into the EcoRI site of plasmid pSER. Bacterial cultures containing plasmids with the same fragment in both orientations were mixed before protoplast preparation. The positive 13-kb EcoRI fragment was subdivided into an 8.6-kb HindIII fragment and a 4.1-kb HindIII–EcoRI fragment. These fragments were cloned by blunt-end ligation into the EcoRI site of plasmid pSER and tested for transformation efficiency against A20-2J. b, The 4.1-kb HindIII–EcoRI fragment was further subdivided as described in the text. Each plasmid was tested at least three times using constructs containing the test DNA fragment in both orientations. For most fragments, only the reverse orientation with respect to gpt transcription is shown. Plasmid nomenclature: see Fig. 1 for the restriction fragments inserted into the EcoRI site of plasmid pSER. The letter R refers to the reverse orientation used for the transformation data shown.

In order to prove more directly that the E_β gene carries an associated enhancer element, we inserted the HB fragment into plasmid pSV-γ2bΔX$_{2/4}$ and analysed the levels of γ2b mRNA in stably transformed A20-2J or J558L cells (pools of several independent colonies.) Plasmid pSV-γ2bΔX$_{2/4}$ contains the coding sequences and transcriptional promoter for the γ2b immunoglobulin heavy chain but lacks the heavy-chain enhancer[8]. High-level expression of the γ2b gene in cells transformed with pSV-γ2bΔX$_{2/4}$ was shown previously to be dependent on the addition of a functional enhancer[8,13].

The levels of γ2b mRNA, determined by S$_1$ nuclease protection, were compared in A20-2J and J558L cells transfected with plasmid pSV-γ2bΔX$_{2/4}$ containing either no inserted DNA, fragment X$_{2/3}$ (heavy-chain enhancer), or the E_β HB fragment in either orientation (Fig. 2). The results show that J558L cells transfected with the construct containing the heavy-chain enhancer contained high levels of γ2b mRNA (lane 2 of Fig. 2c), confirming the results of our previous report[8]. In contrast, no

Hind III
AAGCTTACCAAATCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGCGCAGGTGTG 100

TGTGTGTGTGTGTGTGTGTGTGTGCGCGCGCGCAGGTGTGTGTGTGTGTGTGTGTGTGTGCGCGCGCAGGTGTGTGTGTGTGTGTGTGTGTGTTGTGTGTGCGC

GCGCGTGCGCGTGTGCAGGTGTGTGTGTGTGCAGATTACAAACCTGTGTGTGCAGATGTATTAATAAGTGTGCCTGTATGCACGTGGAGGGCAGAGGTCA

GCATCTGGTGTCTTTCTCAGTTGCTATTAGCCTCCTTATTTTGAGATAGGGTCTATCACAGAGACCTAGAGATTTTGAGCTGGCAGGCCACTGAGCCTTG

Ava II
GGGACCTGTCTGTCTCAGCCACTCCAGAGTTGTGACTAGAAGAGAACACTACGTTTTTTTTTTTTTTTTAATGTGGGTACAGTGTGCCCCAACTTAGGTCCT 500

CATCCTTGTTTGGCATACACCTTACCAATTGAGTCATTTCCCAGCCCCAAGTGGTCAGGTTCTACAGTGTTCACATCATATGCAATTACTGGTCATGGAG

TTGGTCAAATAAGGAAATCAGATTCTGAAAACTAATCTTCCAAATACCTGCTGACGTGGCTTAGCAGGTAAAGCTGGTTTTTTTTTTTTTTTTTTGCCAAA

CTTGAGGAGTTCAAGCCCCAAGTCCCAGCAAGGAGAAAGTGAGTCATGAAAATTGAGCTTTGACCACCACATGTGACAGGCATGCTGTGACATGTGACAC

ATATACACACATACTCAAGCACACACACATGCACACTAGATAAATCCATGTAATAAAAACTTTAAATGTAATAATCTTCCCACTTGGGCCTTTTAGCACA

GTCTGTGATCTGAGCAAGTTCGGATGTTAAGGCAGGAGGATCACATGTTCAAGGCTAACCTAGGCAATTAATGAGTCCTGCCTGAAAGGAGAGAGAAAGG 1000

GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGATCTGGGCCTCTAGCTCAGTGGGAGGCCTTTGCCCAGCATGCTGTGG

Pvu II
TTTTAGGTTCAACCCCTAGTACTTTATTCTGCCCAGTGTTAGTTTAAACTTTAAGGAGGTCAATGCTTTGCCATGGGGCAGCTGACAGGAGATCTAGATG

GGGAAAGAACTTAAACTCTTCCCATATACGATACTAATCTTTAGGGTTGTGAGAAGTCTAGTTCCCTCCCTGAGAATCCACAGTAATTTTAGATCTGACC

TCAGCCTGTTGTGCTAAAAGATAGATGAACACATCTCTCAAGGTGGCCCACCAAGTCAGAGAACTGCTTCAGTCCTGATCCAGCTCAGCGATGCTCTCCA

CACTCCCCAGTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCCTCTCTCAACACTTCAACTGAACCAGACCCTCATCTCCCTAGGATGCCCA 1500

Ava II
GCCTCTGCTCTGAATGTGTCCCCTTGAGGACTGGGTTTTAGATAACAACAGGGACCCTCCTCTGTGCCACTAAAGTCAGTAACAGTTCAGGGGTCAAGCTG

GGAACATCCCTTATGTCAGGACTATACCATGACTAACCTTAATTTTCCATTAGCTTGTTAGAATTTCCCTAGCTGAAGAAGTGCAGACTCCACTGTCCAA

TCAAGAGGCTAAATTCTGCTTCGTCATTAGTTACTAGGGAAGGTTGCTCCTCTAGGCGTCCACAGAAGCACAGCCTTCAGTTCTAGAGTAGGTGGCAACT

TTCCCTGAAACAAACACACTTCAGTCACTTTTAACACCGACACTACTGAGCTGAAGTGCAGTAAGCGCTAGGCTAAAGGTGCTTGGCTGACAGTCTCTTC

CTTCACCTTTGATCTCACACAACTTCAAACACACACCAGCCTTGTTTAGGAAACACTCCCCCAGTGCTCAAACTGAGTAAAAGACTGACCATCCCAGAGC 2000

BamHI
TGGCCCAGCTCTGTGGCCTGATTAAAGTGTTTCAGTTGCAACTGGATCCAGCATTTAAAAAAAAGAAAATAAATTGATCCTTTAAAAAAATAATATATAATT

TATACCCAGAAGAAGTTATATGTGTATACAATGTATTTTGAGCCTATCCACCACCCACTACCTCCTTCCAGCTTCTTTTGGAACCTTCCAATAATGTTTC

CCTTCCCAAATTCATTTCCTCTTTAAACAACAACAACAACAACAACAACAACAAACCAAAAACACACACACAAAAAAAAACAAACAAACAAACAAACA

AACAAAAAACCCAAAGCAAAACCCTTCTCTCCTTTTGTTTGTTTGGTTTTTGTTGTTGTTTATTTGTTTGTTAATTAGAGACAGAATCTTACTATGTAGA

Pvu II
GCAGGCTGACCTTTCTATCTCACAAATATCCAGCTGCCTCTGCCTCCTGAGTGCTGGGATATGAGGCATGGGCCAGCAGCCCAGACTGAGTATCCATGTA 2500

ATGAAGAGAACTGCAAGTTTCAGAAGGGGACCTGCAAACTGAATCTCTAACTAGCAACTGATGATGCTGGACTCCTTTGATGCTGATTGGCTCCCAGCAC

BstXI
TGGCCTTACCCAATCCAGTGGCAAAGCAGTGAATGTCCTGTCTCTTATTATCTTAGCAATGAGTAAAGAGAATTAAGTTACAGTCTGAAGTTTGCCTTCC
                  -26   -20   -10
                  MET Val Trp Leu Pro Arg Val Pro Cys Val Ala Ala Val Ile Leu Leu Leu
CCTCTGACTCCTGTGTCTCCTCTCCTGCAGC ATG GTG TGG CTC CCC AGA GTT CCC TGT GTG GCA GCT GTG ATC CTG TTG CTG
          -1   1
Thr Val Leu Ser Pro Pro Val Ala Leu Val Arg Asp Thr Arg P
ACA GTG CTG AGC CCT CCA GTG GCT TTG GTC AGA GAC ACC AGA C GTAAGTGCACACCTCAGGTGCTGGGATGCTTGGGGTCGGGG

Fig. 3 Sequence of the 5' flanking region and exon 1 of the mouse E_β^d gene. The sequencing strategy is shown in Fig. 1 and was performed by standard procedures[19]. Exon 1 is underlined with a thick line. Boxed sequences include two conserved sequence blocks shared with E_α and the human DR_α genes[5] (nucleotides 2,551 and 2,583), the 'CCAAT' box at 2,610 and the 'TATA' box at 2,646. Short sequences resembling enhancer 'core' elements are underlined and their orientations with respect to E_β transcription are indicated with arrows. Three long stretches of purine–pyrimidine asymmetry are also underlined and begin at nucleotides 13, 1,001 and 1,411. Stretches of alternating purines and pyrimidines are indicated with a dotted line.

copies, it seems that the strength of the heavy-chain enhancer in J558L as opposed to A20-2J is even greater than the differences seen in the intensity of the S_1-protected bands (Fig. 2, lanes 2 and 6). Note, however, that factors such as mRNA stability or differential promoter strength may also affect the steady-state levels of γ2b mRNA in the two cell types. Nonetheless, these results are in agreement with the data obtained from the pSER transformation assay (Table 1) and demonstrate that the heavy-chain sequences function as an enhancer in both B cells and plasma cells, albeit with different strength, while the E_β HB

fragment functions as an enhancer in the B lymphoma expressing class II antigens but not in the myeloma not expressing these antigens. Thus, functioning of this enhancer correlates with the expression of the E_β gene in the terminal stages of B lymphoid cell differentiation.

DNA sequence analysis of the 5' flanking regions of the E_β^d gene revealed no significant similarity to the heavy-chain enhancer (Fig. 3). The enhancer 'core' sequence, $5'GTGG^{AAA}_{TTT}G 3'$ (ref. 14), common to most known viral and cellular enhancers, occurs once in this region around nucleotide 2,150 and is oriented in

the direction opposite that of E_β transcription. Three 'core-like' sequences, each containing a C residue between the third and fourth G residues of the core consensus sequence, are located between nucleotides 551 and 767. These sequences are spaced approximately 100 bp apart and each successive repeat contains an additional A or T between the third and fourth G residue than the one before it. A fourth 'core-like' sequence containing five A or T residues is located around nucleotide 1,100. All four 'core-like' sequences are contained within the $Hind$III–PvuII (HP) and AvaII–AvaII (AA) fragments which by themselves have no enhancer activity (Table 1).

The most striking feature of the sequence in this region is the dinucleotide repeats beginning with 24 TC repeats near the $Hind$III site. This is followed by a track of alternating purine–pyrimidines which extends for approximately 170 bp with only five single base pair interruptions. Shorter stretches are also found around nucleotides 250 and 800. Such sequences are known to have the potential of forming left-handed Z-DNA[15], however this property is not by itself sufficient for the enhancing activity of the E_β enhancer as these sequences are located within the inactive HP fragment.

There are two additional tracks of purine–pyrimidine asymmetry—a GA repeat between nucleotides 1,001 and 1,054 and a TC repeat between nucleotides 1,411 and 1,450. It is not yet known whether such sequences have a role in E_β enhancer function. It should be noted, however, that the $(TC)_{24}$–$(GA)_{27}$–$(TC)_{20}$ tracts are all contained in the HB fragment (the smallest fragment with full enhancing activity) whereas the inactive HP, PB and AA fragments contain only one or two. Clearly, the individual contributions of the elements described above as well as those involved in the strict cell-type specificity of this enhancer will require much more refined analyses.

The results presented above provide another example of a cell type-specific cellular enhancer element which is likely to play an important part in the regulated expression of the associated gene. It will be interesting to determine to what extent the expression of other eukaryotic genes is controlled by enhancers or similar cis-acting elements such as the upstream activation sequences (UASs)[16] of yeast and the 'modulator' sequences of the sea urchin histone[17], human insulin and chymotrypsin genes[18].

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