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A pre-B- and B cell-specific DNA-binding protein, EBB-1, which binds to the promoter of the V_{preB1} gene*

The V_{preB1} protein is thought to be expressed on the surface of pre-B cells in association with λ_5 and μ heavy chain, and to play an important role on B cell differentiation. The expression of V_{preB1} and λ_5 is pre-B cell specific, and regulated at the initiation of transcription. We have identified at least two sequence-specific DNA-binding proteins which bind to the region -191 to -74 of the promoter of the mouse V_{preB1} gene. These DNA-binding proteins also bind to the promoter of the mouse λ_5 gene. One of the two DNA-binding proteins, called EBB-1, is restricted to pre-B and B cells, but not detected in plasma cells, T cells and cells of other lineages. Transient transfection analysis of reporter constructs revealed that the binding sites of these proteins play a significant role in the activity of the promoter, especially the binding site of EBB-1. Taken together these results suggest that EBB-1 might be one of the crucial factors which regulates a series of intracellular events in B cell differentiation.

1 Introduction

B cell differentiation from pluripotent stem cells, progenitors and precursors to antigen-reactive B cells and to Ig-secreting plasma cells is characterized by sequential expression of surface markers and by successive rearrangements of the gene segments of the H and L chain loci of Ig [1–3]. V_{preB}, λ_5 and mb-1 are three B lineage-related genes which have been found to be expressed in pre-B cells (V_{preB} and λ_5, [4–6] or in pre-B cells and B cells (mb-1; [7, 8]) but not in plasma cells or other hemopoietic cells. The proteins encoded by V_{preB} and λ_5 can form an L chain-like structure and be expressed on the surface of pre-B cells as proteins in association with μ H chains [9–13]. These genes are turned off when pre-B cells rearrange L chain genes and become sIg^+ B cells. mb-1 and, at least one other gene, B29 [14] continue to be expressed in B cells, only to be turned off in plasma cells.

The expression of V_{preB} and λ_5 has been found to be regulated at the stage of initiation of transcription [15]. We have begun a search for DNA-binding proteins which interact with specific DNA sequences in the promoter region of V_{preB1}, and therefore might be candidates for B lineage and differentiation stage-specific transcription factors.

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Abbreviations: CAT: Chloramphenicol acetyltransferase **EMSA:** Electrophoretic mobility shift assay

Here, we show that at least two DNA-binding proteins interact with the promoter of the V_{preB1} gene. One of these DNA-binding proteins, termed EBB-1, is specifically detected in early stages of the B cell lineage. By introduction of EBB-1 binding-site mutations we have also shown that binding of the EBB-1 protein is essential for the activity of the V_{preB1} promoter in pre-B cells.

2 Materials and methods

2.1 Plasmid constructions and oligonucleotides

All recombinant DNA work was done according to standard procedures [16]. An ~800-bp Bam HI-Pst I fragment which contains the 5′ flanking region of the mouse V_{preB1} gene was isolated from 7pB12-2 [6]. After changing the Bam HI site to Pst I site with Pst I linker, pUC VpP was constructed by inserting this fragment into the Pst I site of pUC18. The Bgl II-Bsm I fragment was prepared by digestion of pUC VpP with Bgl II and Bsm I.

Oligonucleotide pairs of the V_{preB1} promoter (oligo1, -191 to -151; oligo2, -150 to -111; oligo3, -110 to -74; oligo4, -170 to -131; oligo5, -130 to -91; oligo5-5, -119 to -91), of the λ_5 promoter (oligo6, -185 to -150; oligo7, -149 to -113; oligo8, -112 to -76; oligo9, -75 to -40; oligo10, -166 to -131; oligo11, -130 to -94; oligo12, -93 to -58) and of the promoter of the sea urchin late histone gene H2A-2.2 (5′-TGTGACGCAG-CGGTGGGTGACGACT-3′) were kindly synthesized by Dr. Hansruedi Kiefer, Basel Institute for Immunology, Basel, Switzerland. All of them have Bam HI ends, except for oligo5-5 which has Hind III ends. The hybridization condition of complementary single-stranded synthetic oligonucleotides is as follows. The same amounts of single-stranded oligonucleotides were annealed by heating at 85 °C for 10 min, followed by cooling to room temperature and leaving at 4 °C for 1 h. The amounts of synthetic double-stranded oligonucleotides used as competitors in the electrophoretic mobility shift assay (EMSA) were measured by ethidium bromide fluorescence quantitation [16]. pUC oligo5 was constructed by introducing oligo5 into

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the Bam HI site of pUC18. The plasmid containing synthetic octamer is a kind gift from Dr. Wang, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan, and contains the sequence 5′-CTCTAGAGAAAT-GCAAATTATCTAGAG-3′, subcloned into the Hinc II site of pUC8. The 57-bp of Eco RI-Hind III fragment was used as a probe of EMSA.

pUC-CAT and L-CAT-E [17] were kind gifts from Dr. I.-L. Martensson, Uppsala, Sweden. pUC-CAT was constructed by subcloning the chloramphenicol acetyl transferase (CAT) gene into the Pst I site of pUC18. L-CAT-E contains a 1.5-kb κ L chain promoter and Xba I-Eco RI fragment of the mouse H chain enhancer upstream and downstream of the CAT gene, respectively. Both ends of the Xba I-Eco RI fragment of the heavy chain enhancer have been changed to Bam HI sites. -657 VpP CAT was constructed by inserting the ~ 800-bp of Pst I fragment of pUC VpP into the Pst I site of pUC-CAT. Two different base-substituted mutants of the promoter of V_{preB1} gene were constructed in the context of -657 VpP CAT as follows. A fragment containing a 5-base exchange 5′-GATCTGCTTATTTGGGGCTCAGCCTCT-CAAGGGGAGTaagctTCACTCTCCC-3′, corresponding to the Bgl II-Sau I region was synthesized. Substituted bases are shown by small letters. After digesting pUC VpP by Bgl II and Sau I, the mutated Bgl II-Sau I fragment was replaced. Thereafter the ~ 800-bp of the Pst I fragment was cut out from the construct and inserted into the Pst I site of pUC-CAT giving C1C2HVpPCAT. As for the other mutant, a fragment containing the 9-base exchange 5′-TC-AGGGAGACAAGCCCGCAGTCCTcGaTTaTGGaCtA-TcAgcaTCCAGCAGGTGCTTCCTCCCAGATGCT-3′ corresponding to the Sau I-Bsm I region was synthesized. Thereafter the Sau I-Bsm I region of pUC VpP was replaced by the mutated fragment in the same way and ~ 800-bp of the Pst I fragment was inserted into the Pst I site of pUC-CAT giving EBB1MVpPCAT. VpPCATE, C1C2HVpPCATE and EBB1MVpPCATE were prepared by introducing the H chain enhancer into the Bam HI site of VpPCAT, C1C2HVpPCAT and EBB1MVpPCAT.

2.2 Cell lines

All the cell lines, described previously [6], were grown in Iscove's modified DMEM, supplemented with 5% FCS (Gibco Laboratories, Grand Island, NY), 100 U/ml of penicillin-streptomycin (Gibco), 2 mM L-glutamine (Gibco) and 5×10^{-5} M 2-ME, except for an embryonic stem cell line CCE (TG19), which was a kind gift of Dr. M. V. Weil, Basel Institute for Immunology, Basel, Switzerland.

2.3 Transfection and CAT assay

The 230–238 pre-B cell line (2.5×10^5 cells) was transfected using a DEAE-dextran method and 2 days later CAT assay was done according to the protocol described by Martensson et al. [17]. Radioactivity of acetylated and nonacetylated chloramphenicol was measured using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and the ratio of acetylated *vs.* total chloramphenicol was calculated (conversion ratio).

2.4 Preparation of nuclear extracts

Preparations of middle-scale nuclear extracts ("midi-extracts") were performed according to the method of Dignam et al. [18]. Preparations of small-scale nuclear extracts ("mini-extracts") were performed according to the method of Schreiber et al. [19]. Protein concentrations of "midi-extracts" were measured by the Bradford assay [20].

2.5 Methylation interference assay

The methylation interference assays were performed according to the method described by Rosales et al. [21], with the following modification. Methylated DNA probe, dissolved in 20 μl of TE, was mixed with 160 μg of 18–81 crude nuclear extracts and 20 μg of poly(dI-dC) in a 400-μl of reaction volume. Following EMSA DNA samples were eluted, cleaved with piperidine and electrophoresed on a 12% denaturing polyacrylamide gel.

2.6 EMSA

EMSA was performed essentially according to the method described by Schreiber [19]. DNA was end-labeled using the large fragment of DNA polymerase I. [^{32}P]labeled DNA (1.5×10^4 cpm) was incubated with 1–6 μg of poly(dI-dC) (Pharmacia, Uppsala, Sweden) and nuclear extracts, which was added last, in a 20-μl reaction volume containing 4% Ficoll, 20 mM Hepes pH7.9, 50 mM KCl, 1 mM EDTA, 1 mM DTT and 0.25 mg/ml BSA (BRL, Bethesda, MO). After incubation at room temperature for 15 min, samples were loaded onto a 4% polyacrylamide gel (30 : 1 cross-linking ratio), which was pre-run for 2 h at room temperature at 140 V in 0.25 × TBE. Samples were electrophoresed at 140 V for 60–90 min. Gels were dried and exposed to X-ray film (Kodak, Rochester, NY). Competition experiments were done by mixing competitor DNA with the binding reaction before adding nuclear extracts.

3 Results

3.1 Nuclear proteins binding to the promoter of the V_{preB1} gene

The region 5′ of the V_{preB1} gene between positions -191 and -74 has been found to be involved in the transcription of the gene in pre-B cells [12, 15]. We therefore decided to search for nuclear proteins which could bind to this region.

Extracts of nuclear proteins were made from the pre-B cell line 18–81 and tested in EMSA with the Bgl III-Bsm I fragment (118-bp between position -191 and -74) of the 5′ region of the V_{preB1} gene. At least three sequence-specific bands (C1, C2 and C3) were detected (Fig. 1 A, lane 1), which could be competed by an excess of cold Bgl II-Bsm I fragments. Two additional bands above C1 were also detected, but the uppermost band appeared to be binding nonspecifically, since it could not be competed by the cold DNA fragment. The second faint band from the top was not reproducibly detected. To define more closely the binding

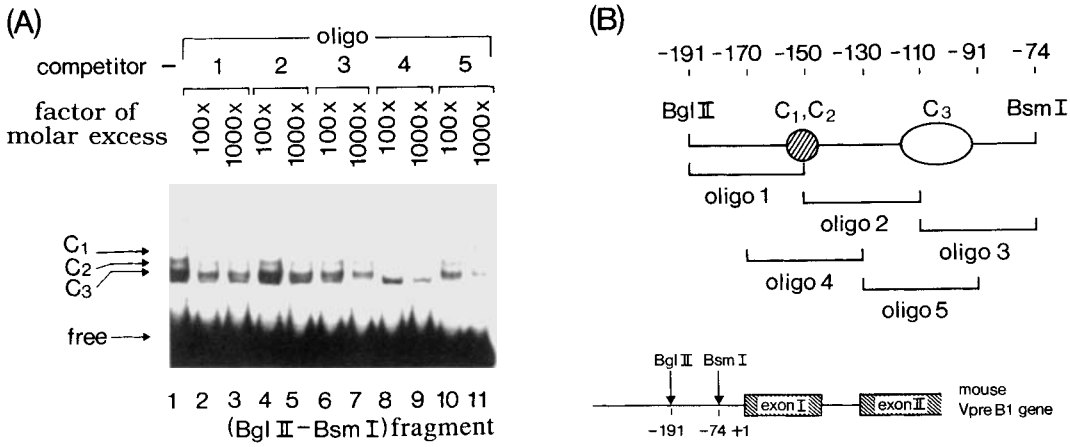

Figure 1. Competition of bindings of C1, C2 and C3 to the promoter of V_{preB1} gene by oligonucleotides. (A) EMSA using the Bgl II-Bsm I fragment as a probe was done as described in Sect. 2.6. 18-81 "midi-extracts" (4 μg) and 6 μg of poly(dI-dC) were used. Bands of protein-DNA complexes and free DNA are shown by arrows indicated with C1, C2, C3 and free. Lanes 2–11 contain competitor DNA in 100- or 1000-fold molar excess. (B) Five oligonucleotides (oligo1, oligo2, oligo3, oligo4 and oligo5) used as competitors are shown by brackets. Positions of ends of these synthetic oligonucleotides are shown by numbers (see Sect. 2.1). Binding sites proposed for C1 and C2 are depicted by circles with shaded lines. The binding site of C3 is depicted by an open ellipse. A scheme of the mouse V_{preB1} genome is also shown. + 1 indicates the major transcription starting site. The positions of restriction enzymes Bgl II and Bsm I (shown by arrows) from the transcription starting site are indicated (-191 and -74).

sites of these three DNA-binding proteins, five oligonucleotides, each around 40-bp in length (referred to as oligo1 to 5) were synthesized. Oligos1, 2 and 3 are non-overlapping and cover the region of the Bgl III-Bsm I fragment. Oligo4 overlaps with oligos1 and 2, while oligo5 does so with oligos2 and 3 (Fig. 1B).

Fig. 1A shows a competition experiment using these oligonucleotides as competitors. Bindings of C1 and C2 to the Bgl II-Bsm I fragment were competed only by oligo4, but not by oligos1 and 2. C1 and C2, therefore, could bind to the area between oligos1 and 2. As for the relationship between C1 and C2, C1 and C2 seem to recognize around the same region. Whether C1 is dimer of C2 or C1 and C2 are different DNA binding proteins with the same binding specificity remains to be investigated. Since there were almost no differences of the intensities of C1 and C2 in EMSA using different quantities of nuclear extracts, it is unlikely that C1 is a dimer of C2 (unpublished data). The exact binding sites of C1 and C2 will be the subject of future investigations. C3 was competed by both oligos3 and 5, but not by oligo2. This indicates that the binding site of C3 might be in the region common to oligos3 and 5. To investigate the tissue specificities of C1, C2 and C3, oligo4 and oligo5 were used as probes in EMSA.

3.2 Tissue-specific binding of C3

Oligo5 bound C3 only, and this binding was competed by an excess of oligo5, less well with oligo3, and not with

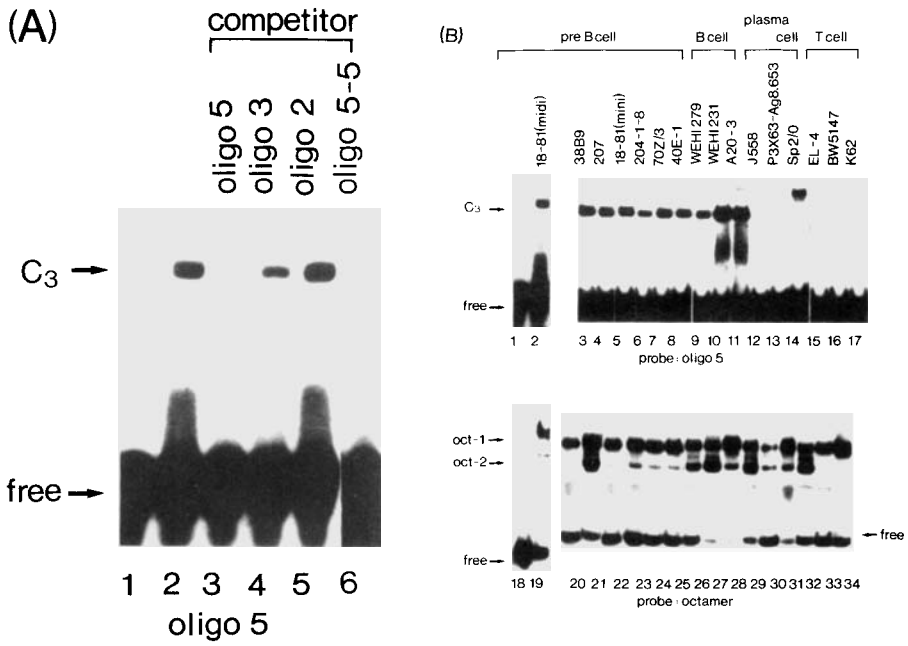

Figure 2. Tissue-specific binding of C3. (A) EMSA was performed using oligo5 as a probe. 18-81 "midi-extracts" (4 μg) and 2 μg of poly(dI) · poly(dC) were used. Lane 1: without nuclear extracts, lanes 2–6: with nuclear extracts. Lanes 3–6 are competition assays. Competitors used are shown above each lane. The amount of each competitor is 100-fold molar excess. Bands of DNA-protein complex and free DNA are shown by arrows indicated with C3 and free, respectively. (B) EMSA was performed using oligo5 and octamer as probes. Nuclear extracts from various lymphoid cell lines were used as shown above each lane. Lanes 1 and 18: without nuclear extracts. 18-81 "midi-extracts" (4 μg) and 2 μg of poly(dI-dC) were used in lanes 2 and 19. Mini-extracts (4 μl) and 1 μg of poly(dI-dC) were used in the other lanes. Positions of C3, Oct-1, Oct-2 and free DNA are indicated by arrows.

oligo2 (Fig. 2A). Using 15 cell lines (5 mouse pre-B cell lines, 3 mouse B cell lines, 3 mouse myeloma cells lines, 3 mouse T cell lines and one human pre-B cell line) C3 binding activity with oligo5 was detected only in pre-B and B cell lines (Fig. 2B, lanes 1–17). To ascertain if the nuclear extracts were qualitatively similar, a subcloned synthetic fragment containing the octamer motif was used as a probe in control experiments. The octamer sequence ATTTGCAT, or inverse complement ATGCAAAT, exists in the regulatory regions of many genes including Ig genes, some MHC class II genes [22], small nuclear RNA genes [23] and the histone H2B gene [24], and has been shown to be important for their activity. At least two factors are known to bind to the octamer motif: Oct-1 which is expressed ubiquitously in all lineage cells and Oct-2 which is expressed only in lymphoid cells [25]. All extracts contained similar quantities of Oct-1, and some extracts contained also some Oct-2 (Fig. 2B, lanes 18–34). An additional eight non-B cell lines, including three T cell lines (CAK4.4, CAK1.3, 110TCKneg), two myeloid cell lines (WEH3, P388D1), two fibroblast cell lines (NIH3T3, Ltk$^-$) and an embryonic stem cell line (CCE), were also tested for C3 activity, and all were found to be negative (data not shown). As for C1 and C2, EMSA using oligo4 as a probe and extracts from mouse pre-B cell line 18–81 showed more than three bands (unpublished data).

We conclude from these experiments that the DNA binding activity C3 is selectively expressed in pre-B and B cell lines, but not in plasma cells and T cell lines. Since one human pre-B cell line (207 in Fig. 2B) also expresses C3-like activity, it is likely to be conserved between the two species. We refer to the activity giving rise to the C3 complex as the early B lymphocyte-specific DNA binding protein, EBB-1.

To define the binding site of EBB-1 in more detail, a methylation interference assay was performed and shown in Fig. 3 and the contact points indicated. To determine the boundary of the binding site of EBB-1, EMSA was done using as competitors several lengths of fragments of the oligo5. Twenty-nine bp of oligo5-5 (the sequence is shown in Fig. 5) could bind EBB-1 with almost the same affinity as the oligo5 (Fig. 2A). Oligo5-5 contains all the contacts points determined by methylation interference assay. Therefore oligo5-5 is thought to be minimal EBB-1 binding site.

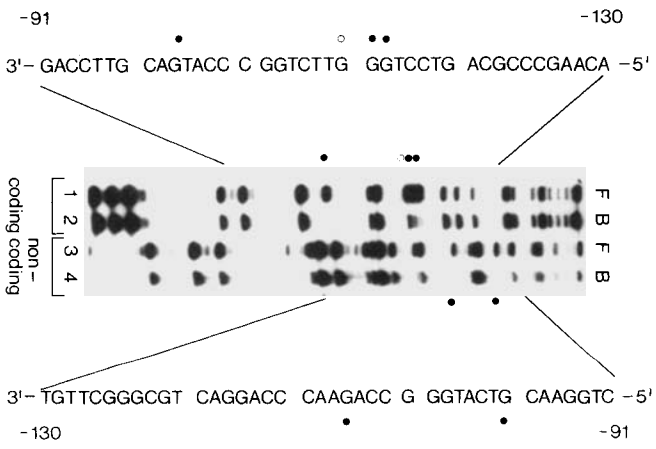

Figure 3. Methylation interference assay of C3. The Eco RI-Bam HI fragment of pUC oligo5, labeled at the 3′ end of either coding (lanes 1 and 2) or non-coding (lanes 3 and 4) strand using the large fragment of the DNA polymerase I, was used as a probe for methylation interference assay. Both free (F) and protein-bound C3 (B) bands were excised from wet gels of EMSA performed with partially methylated probes and were analyzed by 12% denaturing acrylamide gel. Contact points are shown in the sequence of the oligo5. Closed circle(●) and open circle(○) show stronger and weaker protection sites, respectively.

3.3 EBB-1 is a common DNA-binding protein of the promoters of V_{preB1} and λ_5 genes

Since the expressions of both the V_{preB1} and λ_5 genes are pre-B cell specific, binding of the C1, C2 and C3(EBB-1)-derived proteins to the promoter of the λ_5 gene was tested. We used the same strategy as in the case of V_{preB1} gene. Seven oligonucleotide pairs, each around 40-bp in length (referred to as oligo6 to 12), were synthesized. Oligo 6, 7, 8 and 9 are non-overlapping and cover the region of Sau I-Nco I fragment of the λ_5 promoter [5]. Oligo 10 overlaps with oligo6 and 7, oligo11 with oligo7 and 8 and oligo12 with oligo 8 and 9 (Fig. 4B). Fig. 4A shows that binding of C3 (EBB-1) to the Bgl II-Bsm I fragment of the V_{preB1} promoter was competed by oligo6 and weakly by oligo9 and that binding of C1 and C2 to the fragment was competed by oligo7, indicating the promoter of λ_5 gene has at least one binding site for C1 and C2, and possibly two for C3 (EBB-1), although their affinities might be different. After subcloning oligo6 of the λ_5 gene into pUC18, a methylation interference assay was performed in the same way as for the oligo5 of V_{preB1} gene (data not shown). Contact points are shown in Fig. 5.

Since Barberis et al. reported a DNA-binding protein, BSAP, with a similar cell type distribution as that of EBB-1, a recognition sequence derived from the H2A-2.2 gene was used as a competitor [26]. The binding of C3 was also competed by the fragment of H2A-2.2 gene. To investigate further the relationship between EBB-1 and BSAP, EMSA was done using the oligo5 of V_{preB1} gene, the fragment of H2A-2.2 gene and the oligo6 of λ_5 gene as both probes and competitors (Fig. 4C). When the fragment of H2A-2.2 gene was used as a probe, one major band, which probably corresponds to BSAP of Barberis et al., was detected and competed by the fragments of V_{preB1} and λ_5 genes (Fig. 4C, lanes 5–8). In the same way, when the oligo5 of V_{preB1} gene was used as a probe, only the binding activity due to EBB-1 was detected and competed by the fragments of λ_5 and H2A-2.2 genes, indicating EBB-1 can bind to the sequences of the promoters of λ_5 and H2A-2.2 genes (Fig. 4C, lanes 9–12). It is also true of the factor(s) causing a sequence-specific binding activity when the oligo6 of the λ_5 gene was used as a probe (Fig. 4C, lanes 1–4). These experiments suggest that EBB-1 may correspond to the BSAP activity previously identified as binding to the promoter of the sea urchin late histone gene H2A-2.2.

3.4 EBB-1 is indispensable for the promoter activity of V_{preB1} gene

To test whether C1, C2 and C3 (EBB-1) contribute to the promoter activity of the V_{preB1} gene, we compared the

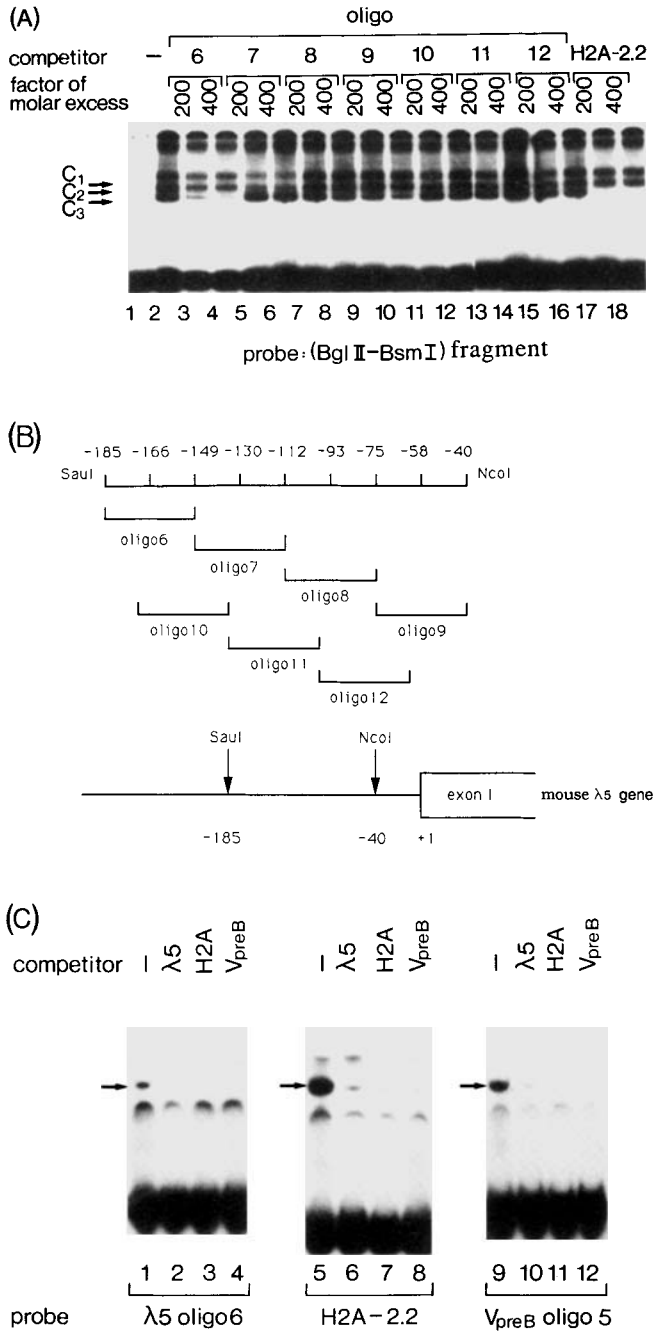

Figure 4. EBB-1 binds commonly to the promoters of V_{preB1} and λ_5 genes. (A) EMSA. Probe, nuclear extracts and poly(dI-dC) are the same as in Fig. 1 A. Competitors and their molar quantities are shown above each lane. Protein-DNA complexes are indicated by arrows (C1, C2 and C3). Lane 1: without nuclear extracts, lane 2: without competitors, lanes 3–18: with competitors. (B) The structure of the promoter region of λ_5 gene is shown. + 1 indicates the major transcription starting site. The positions of restriction enzymes Sau I and Nco I (shown by arrows) from the transcription starting site are indicated (-185 and -40). Seven oligonucleotides (oligo6, oligo7, oligo8, oligo9, oligo10, oligo11 and oligo12) used as competitors are shown by brackets. Positions of ends of these synthetic oligonucleotides are shown by numbers (see Sect. 2.1). (C) EMSA. The probes used are shown at the bottom. 18-8 "midi-extracts" (4 μg) and 2 μg of poly(dI-dC) were used. Lanes 1,5 and 9: without competitors. Competitors used are shown above each lane. The amount of each competitor is 200-fold molar excess. λ_5: λ_5 oligo6, H2A: a fragment of the H2A-2.2 gene containing the binding site of TSAP, V_{preB}: V_{preB1} oligo5.

Figure 5. Sequence comparison of the EBB-1 binding site of the promoters of the V_{preB1} and λ_5 genes. EBB-1 binding sites of the promoters of the V_{preB1} and λ_5 genes are shown. The sequence of V_{preB1} is that of the oligo5-5. The conserved, four external oligonucleotides are shown by arrows. Contact points determined by methylation interference assay are shown (V_{preB1}; see Fig. 3, λ_5 data not shown). Sequence alignment is shown and identical sequences are indicated with slash.

activities of wild type and mutant promoters using an *in vivo* functional assay with the CAT gene as a reporter. For the EBB-1 binding site, 9 bases including the G contact residues were changed to other bases. Since the binding site of C1 and C2 was shown to contain the boundary between oligo1 and 2, five bases were changed in that region. These mutated promoters abolish the bindings of C1, C2 and EBB-1, as ascertained by EMSA using double-stranded

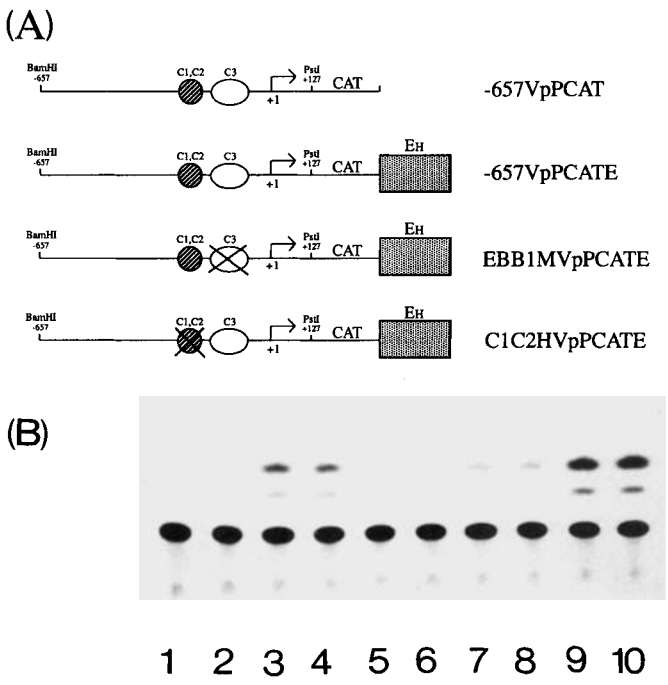

Figure 6. EBB-1 is essential for the promoter activity of the V_{preB1} gene. (A) Four CAT constructs are illustrated with their names on the right. The promoter of the V_{preB1} gene from the Bam HI site to the Pst I site is placed upstream of CAT gene with or without the H chain enhancer. The major transcription starting site is shown by an arrow and given + 1. The positions of the Bam HI and Pst I sites from the starting site are shown by -657 and + 127. The binding sites of C1, C2 and C3 are depicted in the same way as Fig. 1 B. The base substitutions in the binding sites of C1, C2 and C3 are marked by crosses, respectively. (B) The CAT assay was performed using constructs as follows. Lanes 1 and 2; -657 VpPCAT, lanes 3 and 4; -657VpPCATE, lanes 5 and 6; EBB-1MVpPCATE, lanes 7 and 8; C1C2HVpPCATE. In lanes 9 and 10 L-CAT-E was used as a positive control.

synthetic oligonucleotides containing those mutations as competitors (data not shown). The CAT assay was done using the mutants, namely C1C2HVpPCATE and EBB-1MVpPCATE, respectively (Fig. 6 A). Since the 5′ flanking region up to -657 of the V_{preB1} gene does not show enhancer activity, the Ig H chain enhancer was included in the constructs. The expression was almost completely lost when the EBB-1 binding site was mutated (Fig. 6 B, lanes 5 and 6). On the other hand, when the C1 and C2 binding site was mutated, the expression decreased to about one third of the wild type level (Fig. 6 B, lanes 7 and 8). From these data, one can conclude that C1, C2 are important and that C3(EBB-1) is necessary for the promoter activity of the V_{preB1} gene.

4 Discussion

We have identified a pre-B- and B cell-specific DNA binding protein, EBB-1, which is indispensable for the promoter activity of the V_{preB1} gene. Several factors have been found that act as B cell-specific transcription factors. Oct-2 interacts with the sequence ATTTGCAT or the inverse sequence ATGCAAAT present in all the Ig promoters and IgH enhancer and appears to be the chief determinant for B cell specificity of them [27–31]. In the human IgH enhancer, HE-2 and E6 binding proteins have been found [32–34]. NF-κB interacts with the κB motif in the κ L chain intron enhancer and is crucial to the activity of the enhancer [31, 35]. In the B cell lineage, NF-κB appears to become constitutively active during the transition from pre-B cells to B cells and continues to be active throughout B cell differentiation [36], although NF-κB is not only a constitutive transcription factor in mature B cell but also is involved in the inducible expression of genes in different types of cells. The tissue-specific and particularly the B cell-specific expression of MHC class II genes is less well characterized. Two DNA-binding proteins are reported to be B cell specific. One is NF-W1 which binds to 5′ flanking region of E_α gene [37]. The other to A_α [38]. The expression pattern of these two DNA-binding proteins are also developmentally regulated, *i.e.* B cell specific and compatible with the expression pattern of MHC class II genes in the B cell lineage. In the course of the progression of B cell differentiation, Oct-2 is expressed throughout B cell differentiation, NF-κB in B cells and plasma cells, while both NF-W1 and the binding protein of the A_α gene are expressed only in B cells. Since EBB-1 is expressed in pre-B cells and B cells but not in plasma cells, it represents a new category of B cell-specific DNA-binding protein involved in the transcriptional regulation of a different set of genes active in B cell development.

In pre-B cells, it is suggested that V_{preB1} and λ_5 make a complex and are associated with the μ H chain as a surrogate L chain on the surface [13, 39] and the μ H chain is associated with mb-1 (also called B34) [8] in the membrane. Since V_{preB1} and λ_5 are associated with each other [13] and the expression of both genes is regulated coordinately, it is reasonable to think that there are factors which regulate the coordinate expression of both genes. It is quite possible that EBB-1 is one of those factors. Moreover, since the expression pattern of the genes such as mb-1, B29, CD19 [40], Lyb-2 [41] and CD20 [42, 43] is pre-B and B cell specific and compatible with the binding pattern of EBB-1, EBB-1 might also be a vital factor for the synchronous expression of these molecules.

The sequences of the binding sites in the promoters of both V_{preB1} and λ_5 genes are similar but not remarkable (Fig. 5). One aspect of the consensus sequence for EBB-1 binding could be deduced like $CTGG(N)_{18-21}CCAG$ in which the external four nucleotides, shown by arrows in Fig. 5, are conserved in a reversed orientation. They consist of a partial symmetrical structure with a spacing. This structure is reminiscent of the consensus sequence of a transcription factor CTF/NF-I, $TTGGCT(N)_3AGCCAA$ [44].

Independently of our work, Barbaris et al. have reported a pre-B- and B cell-specific transcription factor, BSAP, as a mammalian homologue of a transcription factor of sea urchin, TSAP, which bound to the promoters of the four tissue-specific late histone genes (H2A-2.1, H2A-2.2, H2B-2.1 and H2B-2.2) [26]. However, they could not find out the target gene of BSAP in mammals. From the competition experiments of EMSA, V_{preB1} and λ_5 genes turned out to be targets for BSAP. The sequences of the binding sites of BSAP in the promoters of the four sea urchin late histone genes are quite different from one another and also from the sequences of the EBB-1 binding sites of V_{preB1} and λ_5 genes. Since both EBB-1 and BSAP can bind to those degenerate DNA sequences, they might recognize a particular type of three-dimensional DNA structure. This phenomenon is also seen in the case of Oct-1 and Oct-2. Oct-1 and Oct-2 are reported to bind to target sequences of the *en* and *Ubx* homeo domain proteins of drosophila and to a Mat-a2 binding site of yeast [45]. Those sequences are quite different from the canonical octamer motif.

One should point out that there is no definite data indicating that the same factor binds to the EBB-1 binding sites of the promoters of V_{preB1} and λ_5 genes although EBB-1 can bind to the promoter of λ_5 gene and vice versa. There could be four possibilities. Firstly, they are the same factor, secondly they consist of several subunits and they share the same DNA-binding subunit, thirdly their DNA-binding domains share homology with each other and lastly they are different although they show the same DNA binding specificity. The same argument is true of the relationship between EBB-1 and BSAP. The fact that when EMSA was done using the oligo5 of the V_{preB1} gene, the oligo6 of the λ_5 gene and the fragment of H2A-2.2 gene as probes, only one band was mainly detected (Fig. 4 C), suggests that the first possibility is the most likely.

As described before, EBB-1 is necessary and essential for the promoter activity of the V_{preB1} gene. Whether EBB-1 is sufficient for promoter activity remains to be investigated, although C1 and C2 are also necessary for the maximal activity. Moreover, since the promoter of the V_{preB1} gene does not have any TATA box, the basic elements which determine the transcription starting sites, and could be the targets for C1, C2 and EBB-1 when they function, also remain to be investigated.

There is a discrepancy between the RNA expression pattern of V_{preB1} (pre-B specific) and the binding pattern of EBB-1 (pre-B and B cell specific). This suggests that other factors, such as C1 and C2, which bind to other nearby

DNA sequence elements must necessarily cooperate with EBB-1 to achieve pre-B cell-specific transcription. The fact that the binding sites of C1, C2 and C3(EBB-1) are located in adjacent regions in the promoters of both V_{preB1} and λ_5 genes supports the notion of the cooperative function of these factors. In a limited number of experiments, the EMSA pattern of C1, C2 is different in pre-B and B cells. Thus, protein-protein interactions or other modifications of C1, C2 might repress the activity of EBB-1. It is also possible that there might be other factors which do not bind DNA but nonetheless mediate the function of EBB-1 specifically in pre-B cell.

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