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A Novel Enhancer, the Pro-B Enhancer, Regulates Id1 Gene Expression in Progenitor B Cells

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The helix-loop-helix (HLH) Id proteins have been reported to function as inhibitors of various differentiation programs. The HLH motif mediates dimer formation between Id and the basic HLH transcription factors. Since Id proteins lack the basic region responsible for DNA binding, the heterodimers cannot bind to DNA. Id proteins have also been found to be involved in early B-cell differentiation. They are expressed at high levels in progenitor B cells (pro-B cells), and the expression is diminished in pre-B cells and mature B cells. This expression pattern correlates inversely with basic HLH protein activity and immunoglobulin enhancer function in B-cell development. Regulation of Id expression may play an important role in transcriptional control of immunoglobulin genes and therefore in B-cell differentiation. We have characterized the regulatory elements of the Id1 gene. Using stable transfectants, transient transfection, and mobility shift assays, we have identified an 8-bp element designated PBE (pro-B enhancer) downstream of the Id1 gene that is responsible for a pro-B-cell-specific enhancer activity. A pro-B-cell-specific protein complex was found to bind to the 8-bp PBE element. Substitution mutagenesis at this binding site showed that it is indeed of functional importance in regulating the pro-B-cell-specific expression of the Id1 gene.

Early B-lymphoid-cell differentiation is marked by a sequential rearrangement and activation of the immunoglobulin (Ig) genes (27). The developmental stages of B lymphocytes include the progenitor stage, at which B cells (progenitor B cells [pro-B cells]) have Ig genes in an unrearranged and transcriptionally inactive state, the pre-B stage, at which cells undergo rearrangement and expression of the Ig μ heavy-chain locus, and the mature B stage, at which subsequent rearrangement and expression of Ig light-chain loci result in the synthesis of heteromeric IgM complexes that are displayed on the surfaces of B cells. Although many regulatory proteins have been demonstrated to participate in these rearrangement and transcription events, the molecular mechanism that governs such an orderly process of B-cell differentiation is not fully understood.

A family of transcription factors known as basic helix-loop-helix (bHLH) proteins has been found to play a key role in various differentiation programs, including the differentiation of B lymphocytes (11, 16, 18, 36, 38). These transcription factors contain a basic region responsible for DNA binding and a helix-loop-helix domain mediating dimerization among the bHLH proteins (9, 22, 23). The bHLH proteins bind to an E-box sequence that is present in the enhancers of both Ig heavy-chain and κ light-chain genes, as well as in the regulatory sequences of other cell-type-specific genes. While a B-cell-specific bHLH protein has not been identified, the E12/E47 and E2-2 proteins, which are expressed in many cell types and at different developmental stages, have been shown to bind to the E-box sequence as homodimers in pre-B cells and mature B cells (3, 12, 15, 24). Furthermore, Schlissel et al. have demonstrated that overexpression of E47 in a pre-T-cell line results in the initiation of B-cell differentiation as reflected by the D-to-J rearrangement and the appearance of the Iμ sterile transcript of the Ig heavy-chain gene (30). Recently, it has been shown that B-cell development is completely blocked in mice carrying a null mutation of the E2A gene (encoding E12/E47) (4, 39). These results have led to the conclusion that the bHLH proteins play an essential role in B-cell differentiation.

The unrestricted patterns of E12/E47 and E2-2 expression make it necessary to establish regulatory mechanisms that inactivate these proteins at the stages at which they are not to function. A group of Id proteins can serve this purpose. Id proteins are members of the helix-loop-helix family that lack the basic region (5, 6, 35). They can form heterodimers with E12/E47 or E2-2, but these dimers do not bind DNA. Therefore, Id proteins act as dominant negative regulators of bHLH factors. The expression patterns of Id genes correlate inversely with bHLH activity and Ig enhancer function in B-cell development (26, 35, 37). The expression levels are high in pro-B cells, and they diminish as differentiation proceeds to pre-B and mature B stages. In pro-B cells, in which the Ig genes are neither rearranged nor transcribed, no E-box binding activity is detectable despite the presence of E12/E47 proteins (15), suggesting that E12/E47 may be sequestered by Id proteins. Transgenic mice that constitutively express the Id1 gene in B cells have a severe defect in B-cell development (34). It is thus apparent that the down regulation of Id genes is crucial for B-cell development to proceed, and mechanisms that control Id gene expression may play an important role in regulating B-cell differentiation.

In this report, we elucidate the mechanisms regulating Id1 gene expression during B-cell differentiation. The Id1 gene is regulated at the transcriptional level as determined by nuclear run-on assays (33), and our current results also support this notion. We have characterized the regulatory elements of the Id1 gene and identified a pro-B-cell-specific enhancer located approximately 3 kb downstream from the gene. Deletion analysis narrowed down the enhancer activity to a 74-bp region. A pro-B-cell-specific protein was found to bind to an 8-bp enhancer sequence as determined by electrophoretic mobility shift assays (EMSAs). Subsequent mutagenesis showed that this 8-bp enhancer element, named PBE (pro-B enhancer), is required for full expression of the Id1 gene in pro-B-cell lines.

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MATERIALS AND METHODS

Cell lines. The mouse B-cell lines LyD9 (20, 25), Ba/F3 (25), PD31, 70Z/3, and WEHI-231 were grown in RPMI medium supplemented with 10% fetal bovine serum and 50 μM β-mercaptoethanol. The medium for LyD9 and Ba/F3 was also supplemented with 10% WEHI-3-conditioned medium as a source of interleukin-3. The mouse F9 embryonic carcinoma (32) and C2C12 myoblast (American Type Culture Collection, Rockville, Md.) cell lines were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. 70Z/3 cells were induced to differentiate by treatment with 20 μg of lipopolysaccharide (LPS) per ml for 24 h.

Stable transfection, RNA isolation, and RNA PCR. LyD9 and WEHI-231 B cells were stably transfected with plasmid constructs by electroporation, and positive clones were selected for neomycin resistance (1). Cytoplasmic RNA from these clones was isolated as described previously (29). The RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Gibco BRL) by using random hexamer oligonucleotides. The cDNA was then amplified by PCR with Id1-specific primers (1, 19). These primers were as follows: upstream, CCAGTGGCAGTGCCGCAGCCGCTGCAGGC, and downstream, GGCTGGAGTCCATCTGGTCCCTCAGTGC. The cDNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also amplified as a control for the amount of total cDNA present in each sample. The GAPDH primers were as follows: upstream, ATGGTGAAGGTCGGTGTGAACGGATTTGGC, and downstream, GCATCGAAGGTGGAAGAGTGGGAGTTGCTG.

Transient transfection and assay of luciferase activity. Transfections of luciferase reporter plasmids into Ba/F3, LyD9, PD31, and WEHI-231 B cells were performed by the DEAE-dextran method (1). Cells were harvested and assayed for luciferase activity 2 days after transfection by using a luciferase assay kit (Promega, Madison, Wis.). The production of photons by this reaction was measured with a luminometer (EG&G Berthold, Nashua, N.H.). The relative activities of the constructs are presented as degrees (fold) of activation. They were calculated by dividing the number of luciferase arbitrary units for each construct by that for 6/0. The relative activities of the constructs bearing the c-*fos* minimal promoter were calculated by dividing the number of luciferase units for each construct by that for pfLUC. The data shown represent the averages of those from at least three independent experiments with standard deviations.

EMSA. Crude nuclear extracts from different cell lines were prepared according to a Nonidet P-40 lysis procedure (31). Mobility shift assays were carried out by using a 97-bp restriction fragment as a probe. To prepare the probe, the *Sal*I (engineered)-*Hpa*II-digested fragment from a plasmid which contains the putative enhancer site was labeled at the *Sal*I site by incorporation of [α-^{32}P]dATP, [α-^{32}P]dCTP, dGTP, and dTTP by using the Klenow fragment of DNA polymerase I. Binding reaction mixtures contained 5 μg of nuclear extracts, 10^4 cpm of labeled probe, 3 μg of poly(dI-dC) in 25 mM NaCl, 0.5 mM Tris (pH 7.5), 0.25 mM EDTA, and 5% glycerol. The reaction mixtures were incubated at room temperature for 30 min before being loaded on 4% polyacrylamide gels (29:1, acrylamide-bisacrylamide) in high-ionic-strength Tris-glycine buffer (1).

Oligonucleotide W (8) and oligonucleotide 9 probes were labeled by filling in the protruding ends of the complementary oligonucleotide pairs with [α-^{32}P]dCTP and [α-^{32}P]dATP, respectively.

Plasmid constructs. To create the mini-Id1 gene, a partial digestion with *Pst*I was performed on a plasmid containing a 9-kb *Hin*dIII-*Not*I fragment in pBluescript II KS+ (Stratagene, La Jolla, Calif.). The partially digested DNA was size fractionated, religated, and cloned into *Escherichia coli*. Clones that contained a 260-bp deletion in the coding region of the Id1 gene were screened by PCR using primers amplifying the coding region of Id1. The plasmid was then digested with *Xho*I and *Hin*dIII and ligated with a *Xho*I-*Hin*dIII fragment containing the neomycin resistance gene, thus generating construct 6.1/ΔId1. A 6-kb *Not*I fragment containing the 3′ flanking sequence was then inserted in either orientation into the *Not*I site of construct 6.1/ΔId1 to create constructs 6.7/ΔId1 and 6.7R/ΔId1.

For the luciferase reporter constructs, DNA fragments of 6 kb each from 5′- and 3′-flanking regions of the murine Id1 gene were subcloned into a vector, pfLUC (a gift from K. Saksela and D. Baltimore), which contains the luciferase reporter gene. The 5′ fragment was inserted into the *Hin*dIII and *Bam*HI sites immediately upstream from the reporter. This insertion replaced the upstream c-*fos* minimal promoter of pfLUC with the Id1 promoter. The 3′ segment was inserted into the *Xho*I site downstream from the reporter and the polyadenylation sequence. Serial deletions of these inserts were generated by using the available restriction sites as depicted in Fig. 3A and C. Further deletions extending into the B1 enhancer region were constructed by using PCR and available restriction sites (see Fig. 2). The primers for the PCR were as follows: upstream, ACTAGGAGGAACCTCGAGTGGGG, and downstream, CTTTGCAGGTACCTCAGAGC, which created *Xho*I and *Kpn*I sites (underlined) for cloning. The *Sal*I site as well as the substitution mutation in B1s was specifically engineered into the B1 region by recombinant PCR (13). The B1 segment subcloned into pBluescript II KS+ (Stratagene) at the *Bam*HI and *Cla*I sites was used as a template for primary PCRs. The primer pairs for creating the *Sal*I site (underlined) were as follows: upstream, T3 sequencing primer (Promega), and downstream, GTCAGGTCGACATGC, for the "left" PCR; and upstream, GCCCCCAAGGCATGTCGACCTGACCCTTACAGG, and downstream, T7 sequencing primer (Promega), for the "right" PCR. A secondary PCR was then performed by using the primary PCR products as templates and T3 and T7 primers. The primary PCR primers for generating the substitution mutation (underlined) were as follows: upstream, T3 primer, and downstream, CCTGAGTAGCAAGACGTCGGGCCTGGGGCATCC, for the left PCR; and upstream, CCCGACGTCTTGCTACTCAGGGCATTGTGCAAG, and downstream, T7 primer for the right PCR. T3 and T7 primers were then used in the secondary PCR. Various mutations of the B1 enhancer were also subcloned into pfLUC upstream from the c-*fos* minimal promoter by using the restriction sites indicated in Fig. 7.

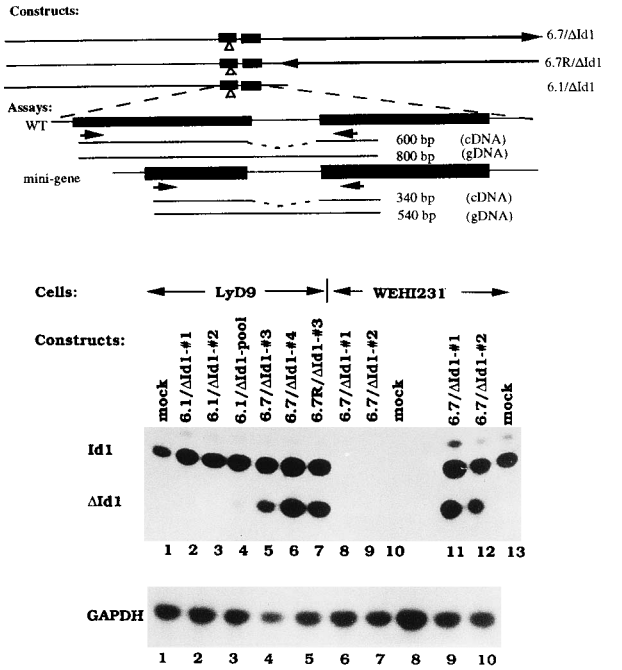

FIG. 1. Expression of the mini-Id1 gene in stable transfectants. (Top) The mini-Id1 gene constructs and an assay for their expression. The filled boxes represent the two exons of the Id1 gene. A 3′ flanking sequence was inserted in both orientations as indicated by the arrows. The pair of PCR primers used is indicated by arrowheads. The sizes of PCR products from cDNA and genomic DNA (gDNA) of the minigene and the endogenous gene are listed. (Bottom) Reverse transcriptase PCR assays for ΔId1 expression in B cells stably transfected with the constructs indicated above each lane. Each lane shows results for individual clones (lanes 2, 3, 5 through 9, 11, and 12) or a pool of nine clones (lane 4) or parental cell lines (mock, i.e., lanes 1, 10, and 13). Each sample was also PCR amplified with primers hybridizing with the GAPDH gene as an internal control to monitor the amounts of cDNAs synthesized. Southern blot analyses were performed by using ^{32}P-labeled probes prepared from DNA fragments of the coding region of the ΔId1 gene and the GAPDH cDNA. Lanes 1 through 10 show products after 22 cycles of amplification. Lanes 11 through 13 show PCR products of 30 cycles of amplification using the same templates as were used for lanes 8 through 10. Products from the endogenous Id1 mRNA, the minigene ΔId1 mRNA, and the GAPDH mRNA are as indicated.

RESULTS

Reconstitution of Id1 expression pattern in B cells by using the cloned Id1 flanking sequences. To determine which flanking sequences of the Id1 gene are necessary to recapitulate the expression pattern of the endogenous Id1 gene, we first created an Id1 minigene, ΔId1, by deleting a 260-bp coding sequence such that the transcript of this gene was easily distinguishable from that of the endogenous gene by reverse transcriptase PCR assays. As diagrammed in Fig. 1, the amplified DNA product from the minigene transcript was 260 bp shorter than the products of the endogenous Id1 mRNA. This product could also be separated from the amplified products of the contaminating genomic DNA. A series of constructs was then generated by using this minigene and various lengths of the Id1

flanking sequences. The transcription initiation site of the Id1 gene has been mapped to a region close to the published cDNA sequence (5) by using primer extension and 5′ rapid amplification of cDNA ends (RACE) protocols (28). Therefore, these constructs all contained about 6 kb of 5′ flanking sequence and different lengths of 3′ flanking sequences as shown in Fig. 1. These constructs were introduced into LyD9 pro-B cells and WEHI-231 mature B cells, and individual cell lines that had these constructs stably integrated were selected by neomycin resistance. The levels of mini-Id1 gene expression in these cell lines were assayed by using reverse transcriptase PCR. As shown in Fig. 1, LyD9 pro-B-cell lines carrying construct 6.7/ΔId1 or construct 6.7R/ΔId1 produced comparable amounts of the Id1 minitranscript and the endogenous Id1 transcript (lanes 5 through 7). Similar results were also obtained when pools of multiple cell lines containing the same constructs were assayed (data not shown). However, LyD9 cells carrying construct 6.1/ΔId1 showed very low levels of minigene expression even though the endogenous Id1 gene was expressed efficiently (lanes 2 through 4). These results suggested that the 6-kb 3′ flanking sequence of the Id1 gene contains essential regulatory sequences, e.g., enhancers, for efficient Id1 expression in LyD9 pro-B cells.

In contrast to pro-B cells, WEHI-231 mature B cells carrying construct 6.7/ΔId1 showed little expression under the same PCR conditions (lanes 8 and 9). It was only when the sensitivity of the assay was increased by performing eight additional cycles of PCR that the DNA products from both the endogenous and minigene transcripts were detected (lanes 11 and 12), indicating that both the endogenous and exogenous Id1 genes are expressed at much lower levels in mature B cells. This result, together with those obtained for pro-B cells, demonstrated that construct 6.7/ΔId1 contains sufficient regulatory sequences to confer the developmental stage-specific regulation of the Id1 gene. Whether the down regulation in mature B cells is achieved by the loss of function of a pro-B-cell-specific enhancer or by the function of a mature B-cell-specific silencer remains to be determined.

Identification of a pro-B-cell-specific enhancer of the Id1 gene. To further characterize the 6-kb 3′ flanking sequence, we generated several plasmid constructs bearing a luciferase reporter gene flanked by 6 kb of 5′ sequence and various regions of the 6-kb 3′ sequence of the Id1 gene (see Fig. 3A). These constructs were then transiently transfected into LyD9 pro-B cells and WEHI-231 mature B cells. The 6-kb 3′ flanking sequence in construct 6/6 was found to cause an increase of expression in pro-B cells and a repression of the gene in mature B cells, as determined by comparing the luciferase activity in cells transfected with construct 6/6 with that in cells transfected with construct 6/0. While further dissection of the 6-kb region (constructs 6/A, 6/AB, 6/BC, 6/B, 6/C, and 6/Dr) failed to localize any significant mature B-cell-specific silencer activity, the same analysis led to the identification of a 700-bp pro-B-cell-specific enhancer element like that found in construct 6/B. This enhancer element resulted in a sixfold increase in luciferase activity in LyD9 cells.

Figure 3B shows results from the analyses of 3′ and 5′ deletion mutants of the 700-bp B region in LyD9 pro-B cells. The detailed restriction maps of the 3′ deletion constructs are summarized in Fig. 2. While 3′ deletions to an *Hae*III site did not abolish enhancer activity (constructs 6/B1 and 6/B2), further deletion in construct 6/B3 abolished most of the enhancer activity. The 5′ sequence to the *Hpa*II site in the enhancer has no apparent enhancer activity, as shown with construct 6/B4, and deletion of a 140-bp 5′ sequence in construct 6/B2 did not affect the enhancer activity. We therefore conclude that the

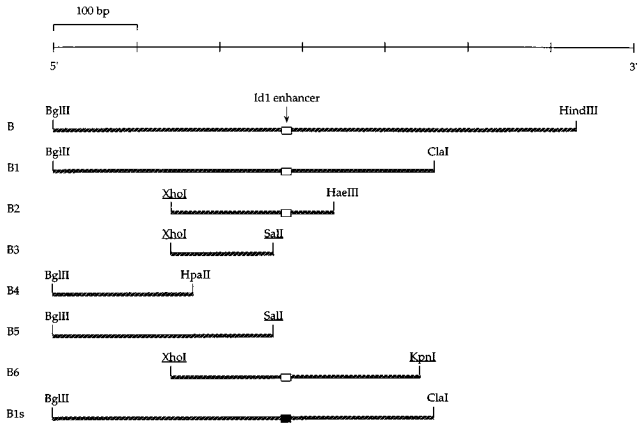

FIG. 2. Restriction map of the Id1 3′ enhancer region B and its mutants. The minimal enhancer segment (PBE) is indicated by open boxes. The substitution mutation of the enhancer element is indicated by the filled box. Restriction sites introduced into the region by recombinant PCR are underlined.

enhancer activity is located in the region between the *Hpa*II site and the *Hae*III site and that the major activity is in the 74-bp region between an engineered *Sal*I site and the *Hae*III restriction site.

We next shortened the 6-kb 5′ flanking sequence into 1-kb and 130-bp fragments, which were subcloned into the luciferase reporter constructs (Fig. 3C), and we asked whether the enhancer element identified above could still activate transcription. First of all, deletions of the 5- or 5.87-kb 5′ flanking sequence resulted in a dramatic increase of expression as determined by comparing the luciferase activity of construct 6/0 with that of 1/0 or that of Spe/0, indicating that certain repressive sequences are present in the 5-kb 5′ flanking sequence. However, this repression is not pro-B-cell specific because a similar increase in activity was also observed in WEHI-231 mature B cells (data not shown). Despite the release of repression by the deletions, the effect of the enhancer was still apparent as shown by constructs 1/B1, 1/B1*, and Spe/B1*. Because construct Spe/B1* contains only 130 bp of sequence upstream of the transcription start site with a putative TATA box, the enhancer probably acts through interaction with proteins binding to the region close to the promoter.

Mapping of the enhancer sequence by an EMSA. In order to delineate the enhancer site, EMSAs (1) were performed. A probe bearing the 74-bp enhancer was incubated with nuclear extracts isolated from various B-lymphoid cell lines as well as other cell types (Fig. 4A). While pro-B-cell lines Ba/F3 and LyD9 share several binding complexes with mature B-cell line WEHI-231, one complex appears to be specific to pro-B cells. This pro-B-cell-specific complex was also not found in nuclear extracts of C2C12 myoblasts, F9 embryonic carcinoma cells, and murine erythroid leukemia cells (Fig. 4A). The C2C12 nuclear extract displayed an apparently similar complex, but it was not identical to the pro-B-cell-specific complex because it migrated slightly faster. Furthermore, it could not be inhibited by the oligonucleotides that eliminated the pro-B-cell-specific binding (data not shown).

To further map the binding site of the pro-B-cell-specific complex, oligonucleotide competitors spanning different portions of the probe (oligonucleotides 9, 11, and 14; Fig. 4E) were used in EMSAs. One of the competitors (oligonucleotide 9) competed efficiently for the binding (Fig. 4B). Oligonucleotide 14 competed for the binding at a reproducibly lower efficiency than did oligonucleotide 9. This was probably due to

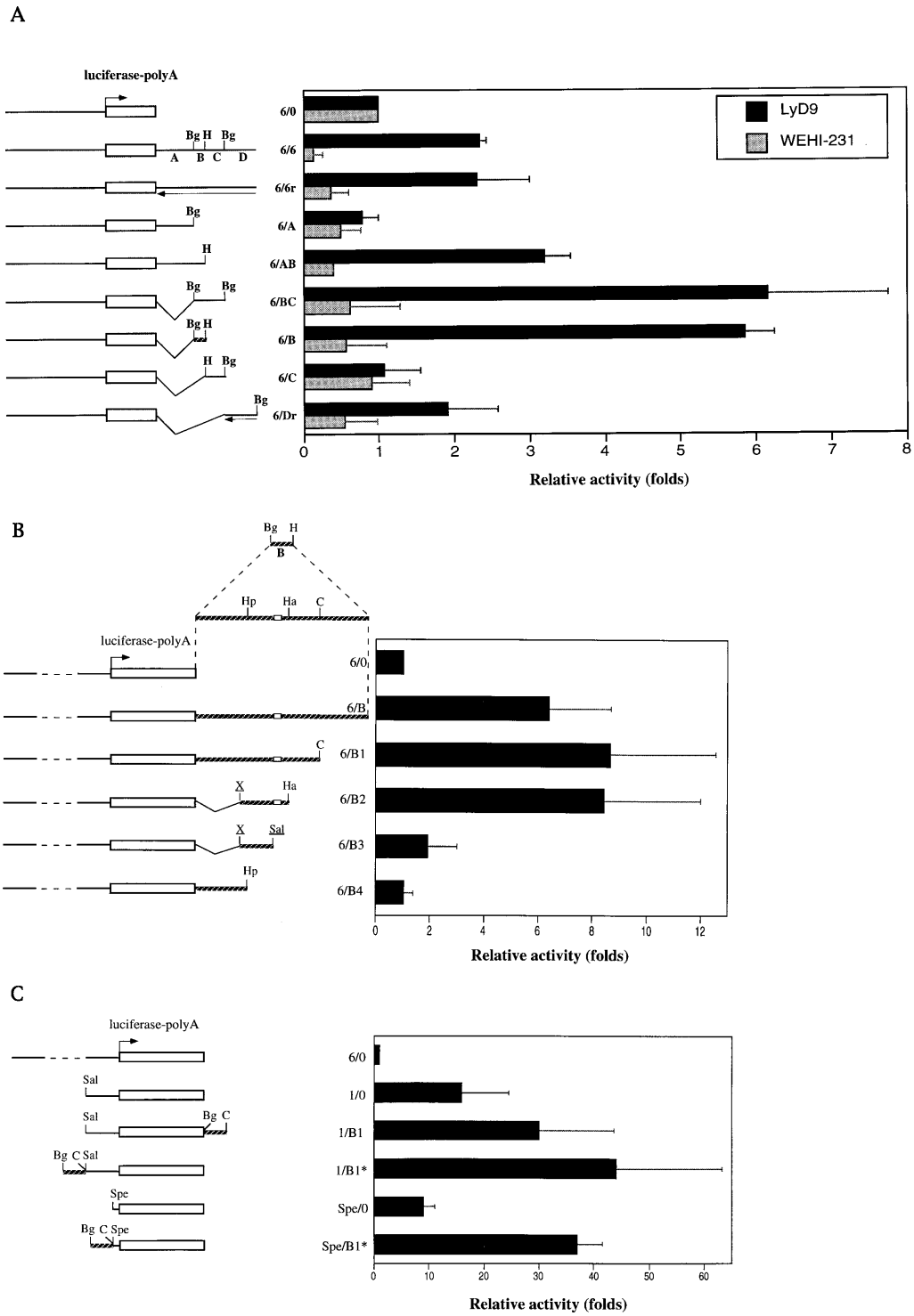

FIG. 3. Transient transfection assay of luciferase reporter constructs in LyD9 pro-B cells and WEHI-231 mature B cells. (A) Both 6-kb 5′ flanking sequences and 6-kb 3′ flanking sequences of the Id1 gene were subcloned into a plasmid containing a luciferase gene. The reporter gene and a simian virus 40 poly(A) sequence are drawn together as an open box. 6/0 is a construct bearing only the 6-kb 5′ DNA sequence upstream from the luciferase gene. Constructions of various deletions into the 6-kb 3′ segment are also shown on the left. (B) Extended functional analysis of the 700-bp (B region) enhancer in LyD9 cells. The 700-bp B region is enlarged to show the restriction sites used. The underlined restriction sites were engineered by PCR. (C) Deletion analysis of the 5′ noncoding sequence of the Id1 gene in LyD9 cells. Plasmid 1/0 is a 5-kb deletion mutant of the 6-kb upstream sequence at the *Sal*I site. Further deletion into the *Spe*I site yielded a 130-bp putative Id1 promoter juxtaposed with the reporter gene (construct Spe/0). The B1 enhancer from construct 6/B1 was subcloned into the two plasmids as depicted. In a typical transfection into LyD9 and WEHI-231 cells, activities of construct 6/0 are in the ranges of 1×10^3 to 2×10^3 and 2×10^3 to 7×10^3 luciferase arbitrary units/min, respectively. The data are presented as described in Materials and Methods. Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; Ha, *Hae*III; Hp, *Hpa*II; Sal, *Sal*I; Spe, *Spe*I; X, *Xho*I.

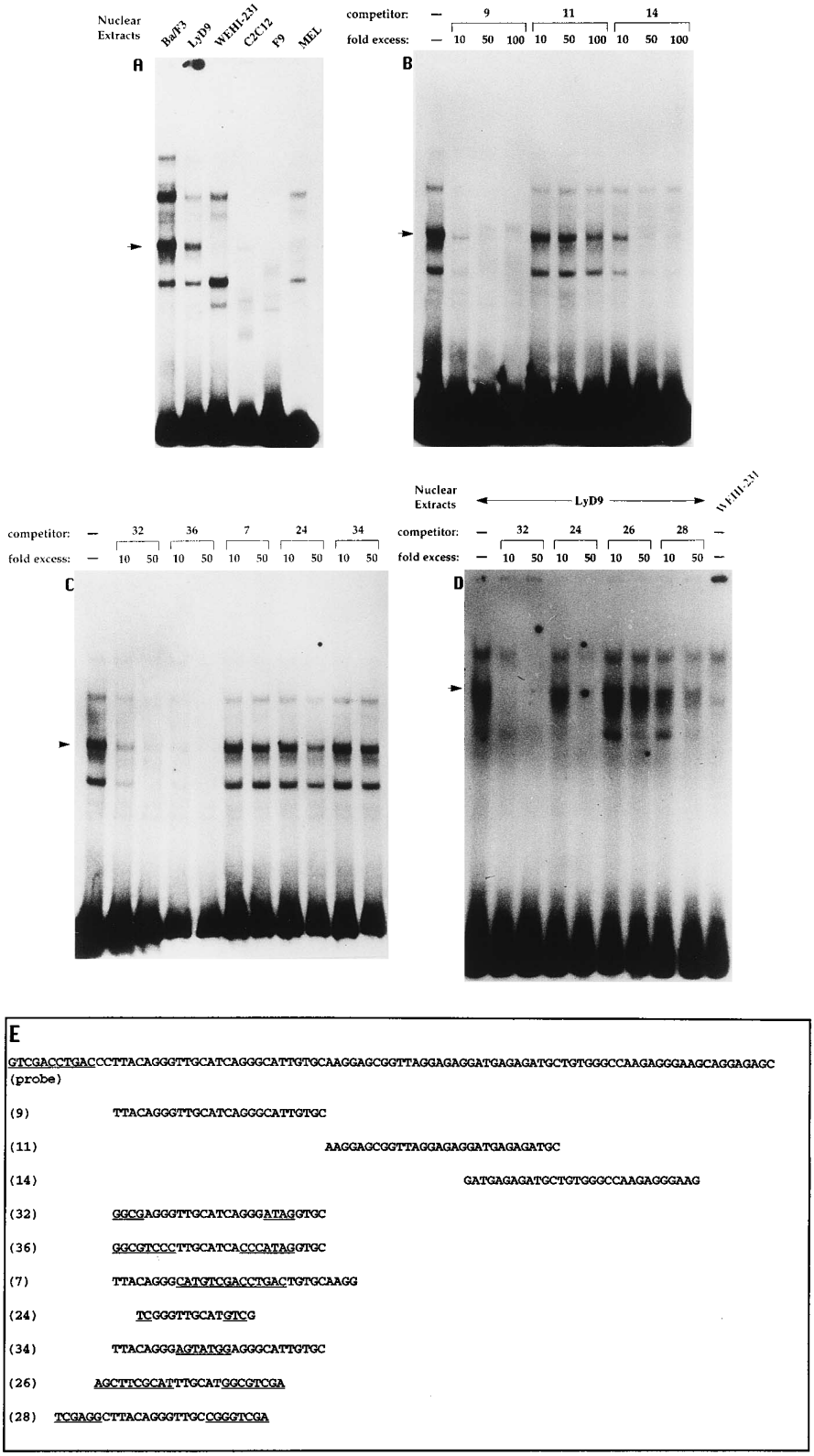

FIG. 4. Characterization of a pro-B-cell-specific enhancer by EMSAs. The sequences of the probes used in the binding reactions are shown in panel E. The 97-bp probe used for panels A through C spans a region downstream from the engineered *SalI* site (Fig. 2). (A) The mobility shift assays were performed with nuclear extracts from various cell lines as listed above each lane. A pro-B-cell-specific complex is indicated by the arrow. (B) Mapping of the pro-B-cell-specific binding site by competition analysis. The binding reactions were carried out with LyD9 nuclear extracts in the presence of a 10-, 50-, or 100-fold molar excess of unlabeled competitors (see Fig. 4E for sequences). (C) Further competition analysis. An EMSA was performed with LyD9 pro-B-cell nuclear extracts. Various competitors were added to the binding reaction mixtures as indicated. (D) A pro-B-cell-specific complex, indicated by the arrow, was also found by using the oligonucleotide 9 probe in the mobility shift assays. Competition analyses were performed with unlabeled competitors as indicated. (E) The top-strand sequences of the probes and the competitors. Mutated sequences are underlined.

a 5-bp similarity between the minus strand of oligonucleotide 14 and the plus strand of oligonucleotide 9 in an 8-bp region that was later identified as the binding site of the pro-B-cell-specific complex (see below). Hence, the primary binding site was confined to a 27-bp segment of oligonucleotide 9. Subsequent competition experiments were performed with oligonucleotides that contain mutations within the 27-bp region (Fig. 4C). Oligonucleotides retaining the sequence TTGCATCA, e.g., oligonucleotides 32 and 36 (Fig. 4E), competed for binding efficiently, whereas oligonucleotides that contain mutations in the entire sequence, such as oligonucleotides 7 and 34 (Fig. 4E), could not compete with the probe for binding. Oligonucleotide 24 contains mutations at the seventh and eighth positions of the sequence shown above, and it can block binding only when used at a higher concentration. We thus concluded that the binding site for the pro-B-cell-specific complex is within TTGCATCA, and we named this site, according to its functional identification, the pro-B enhancer or PBE. The same conclusion can also be reached on the basis of the results of competition experiments performed by using oligonucleotide 9 as a probe (Fig. 4D). By using the probe, a pro-B-cell-specific complex (the top band of the doublet as indicated by an arrow) was found in the LyD9 extract but not in the WEHI-231 extract. It could be completely abolished by competition with oligonucleotide 32 and partially abolished by competition with oligonucleotide 24 at a higher concentration. However, oligonucleotides 26 and 28 did not compete significantly.

When this PBE sequence was compared with sequences in the transcription factor binding site database (10), we found a partial overlap between the PBE site (TTGCATCA) and the W site (GTTGCATC) in the enhancer of the major histocompatibility complex class II gene (8). To determine whether the pro-B-cell-specific binding complex is identical to the B-cell-specific NF-W1 complex described by Dorn et al. (8), we performed an EMSA using the W site oligonucleotide as a probe. Since the probe contained the PBE and W sites (Fig. 5A), both the PBE and NF-W1-binding complexes could be expected. One interesting feature of the NF-W1 complex is that it is LPS inducible in 70Z/3 pre-B cells, which facilitates its identification by EMSA. As shown in Fig. 5A, the NF-W1 complex was dramatically induced in LPS-treated 70Z/3 cells and was also evident in WEHI-231 cells. However, both Ba/F3 and LyD9 pro-B cells did not display the NF-W1 complex. Instead, they exhibited a faster-migrating complex, which could be inhibited by the PBE-containing oligonucleotide 9, suggesting that this complex may be the same as the previously identified pro-B-cell-specific PBE-binding complex. It thus appears that the pro-B-cell-specific complex that we have identified is distinct from the NF-W1 complex.

The PBE site also resembles the AP-1 binding site (TGAN TMA), with a single nucleotide mismatch at the third position (A) of the AP-1 site. We next tested whether this pro-B-cell-specific binding complex belongs to the AP-1 protein family. Competition experiments using two different AP-1 sites were performed (Fig. 5B). None of the oligonucleotides competed for binding, which suggests that the pro-B-cell-specific binding protein may not recognize these AP-1 sites that are bound by various leucine zipper-containing proteins, such as Jun and Fos (7).

The 8-bp PBE site has an enhancer function. The functional significance of the 8-bp PBE sequence was tested by introducing a substitution mutation into construct 6/B1 to generate construct 6/B1s. The mutation (underlined), which changed the 8-bp TTGCATCA sequence into GCTACTCA, abolished most of the enhancer activity in LyD9 pro-B cells as shown in Fig. 6. Similarly, when the same substitution mutation was

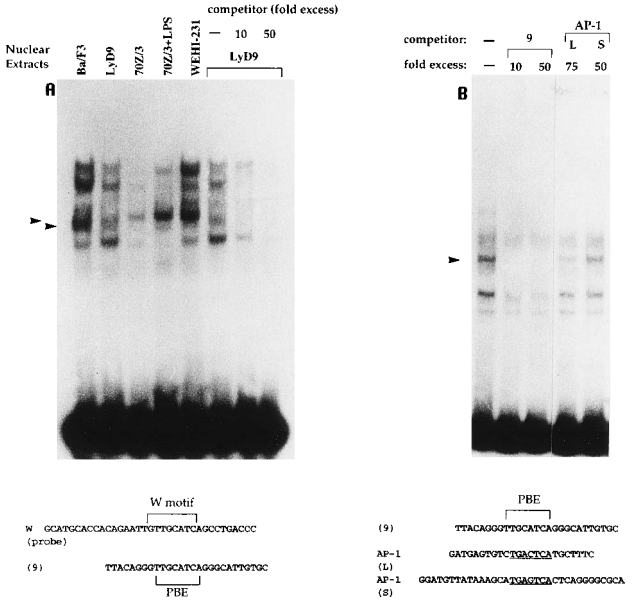

FIG. 5. Different binding characteristics of the pro-B-cell-specific complexes from two known enhancer-binding proteins. (A) Distinct cell-type distributions of NF-W1-binding (arrow) and PBE-binding (arrowhead) complexes. The EMSA was carried out by using oligonucleotide W as a probe. The binding reactions were also performed with LyD9 nuclear extract in the presence of oligonucleotide 9 competitor. (B) Competition analysis with two different AP-1 oligonucleotides, L and S. The 97-bp probe (Fig. 4) was used in the EMSA. The oligonucleotide 9 competitor was included as a positive control.

placed in the context of construct 1/B1* to create construct 1/B1s*, a significant loss of enhancer activity was observed in both LyD9 and Ba/F3 pro-B cells. These data indicate that the 8-bp pro-B-cell-specific binding site is indeed relevant to the enhancer activity.

The Id1 enhancer acts on a heterologous promoter. To test whether the Id1 enhancer can activate transcription from a heterologous promoter, we inserted the 500-bp enhancer fragment B1 immediately upstream of the c-*fos* minimal promoter which drives a luciferase reporter gene in construct pfLUC (Fig. 7). In transient transfection assays, the 500-bp element enhanced the reporter expression in a pro-B-cell-specific manner as found for both LyD9 and Ba/F3 pro-B cells (Fig. 7 and Table 1; construct pfLUC/B1). The substitution mutation brought about approximately 61 and 69% losses of the enhancer activity in LyD9 and Ba/F3 cells, respectively (construct pfLUC/B1s). In LyD9 cells, a deletion mutation removing the 3′ half of the 500-bp segment including the PBE site caused a 72% reduction of the enhancer activity (construct pfLUC/B5). Constructs pfLUC/B6 and pfLUC/B3 contained a 145-bp deletion from the 5′ end of the B1 enhancer fragment (see Fig. 2 for the maps of fragments B5, B6, and B3). pfLUC/B6 retained the enhancer activity, which confirmed that the deleted upstream sequence has no pro-B-cell-specific enhancer activity. pfLUC/B3 lost about 86% of the enhancer activity because of a 3′ deletion which included the PBE element. Together, these data also established the functional relevance of the binding site. Because the minimal promoter of the c-*fos* gene contains only 56 bp of sequence with a TATA box, it is likely that the Id1 enhancer increases the efficiency of transcription from the basal promoter.

The Id1 enhancer does not function in pre-B cells or mature B cells. To confirm the pro-B-cell specificity of the Id1 enhancer, we transfected several relevant constructs into PD31

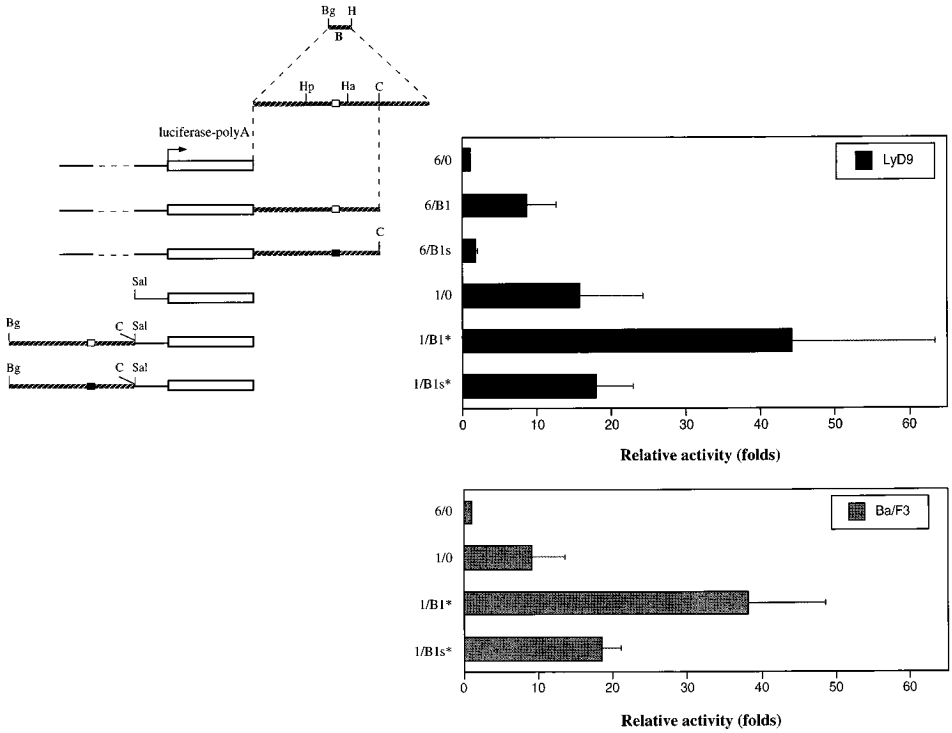

FIG. 6. Mutational analysis of the Id1 enhancer. Transfections were performed with LyD9 (top panel) and Ba/F3 (bottom panel) pro-B cells, and reporter gene expression was assayed. A 13-bp substitution mutation (filled box) was made in the 6/B1 and 1/B1 plasmids, causing a change of the first 5 bp of the 8-bp PBE element from TTGCA to GCTAC. The enhancer is not drawn to the scale of the reporter gene. Construct 6/0 in LyD9 and Ba/F3 cells displayed activities of 1×10^3 to 2×10^3 and 2×10^4 to 8×10^4 luciferase arbitrary units/min, respectively. The data are presented as described in Materials and Methods. Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; Ha, *Hae*III; Hp, *Hpa*II; Sal, *Sal*I.

pre-B cells and WEHI-231 mature B cells, both of which express the endogenous Id1 gene at very low levels. The results are listed in Table 1 in comparison with those for LyD9 pro-B cells. Construct 6/B1 displayed insignificant levels of activity in PD31 and WEHI-231 cells, and mutation in the PBE site had no effect. Similarly, when the B1 fragment was placed upstream of the *fos* promoter in construct pfLUC/B1, its activity was markedly reduced in PD31 and WEHI-231 cells compared with that in LyD9 cells. More importantly, the activities observed in PD31 and WEHI-231 cells were not associated with the PBE site, as suggested by the unchanged activities of the PBE mutant construct pfLUC/B1s.

DISCUSSION

The temporal regulation of Id expression has been found to play an important role in various differentiation programs (17, 21). In B-lymphoid cells, constitutive expression of the Id1 gene under the control of a heterologous promoter results in a blockade of early B-cell development (34). This result suggests

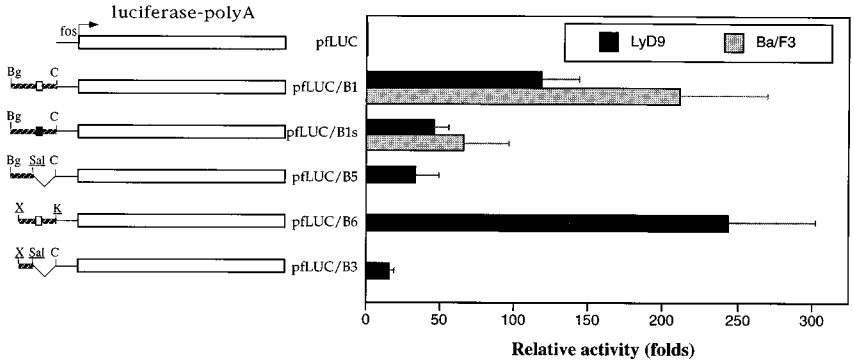

FIG. 7. Functional study of the action of the Id1 enhancer on a heterologous promoter. The B1 enhancer, its deletions, and a substitution mutation were subcloned into a plasmid at the multicloning site upstream from the c-*fos* promoter (single line) and the luciferase gene (long open box). Transient transfection into LyD9 and Ba/F3 pro-B cells and luciferase assays were carried out by using pfLUC, the original plasmid without the Id1 enhancer, as a standard for basal expression of the reporter. The activities of the construct pfLUC were in the ranges of 1×10^2 to 3×10^2 and 2×10^3 to 3×10^3 luciferase arbitrary units/min in LyD9 and Ba/F3 cells, respectively. The data are shown as described in Materials and Methods. Bg, *Bgl*II; C, *Cla*I; K, *Kpn*I; Sal, *Sal*I; X, *Xho*I.

TABLE 1. Transfections of the Id1 enhancer constructs into B-cell lines

Construct	Relative luciferase activity (fold) in the following cell line[a]		
	LyD9	PD31	WEHI-231
6/0	1.00	1.00	1.00
6/B1	8.67 ± 3.88	1.13 ± 0.40	1.50 ± 0.42
6/B1s	1.74 ± 0.31	1.62 ± 0.69	1.51 ± 0.56
pfLUC	1.00	1.00	1.00
pfLUC/B1	118.30 ± 25.59	43.51 ± 3.57	9.68 ± 4.40
pfLUC/B1s	45.70 ± 10.32	42.34 ± 5.17	6.32 ± 2.14

[a] Relative activities of the constructs are shown as described in Materials and Methods.

that the expression of Id genes must be tightly regulated during the course of differentiation. Since Id1 mRNA is present at a high level at the immature stage of B cells and Id1 expression is diminished upon differentiation (35, 37), at least two modes of regulation can be envisioned. The gene can be turned on by pro-B-cell-specific positive elements or shut off by mature B-cell-specific negative elements. Although our initial finding with the luciferase reporter construct 6/6 suggests a mature B-cell-specific repression in the 3′ flanking sequence, we have not been able to map the negative effect to a specific region. Rather, repression appeared to be caused by multiple sequence elements at several sites within the 3′ flanking region of the Id1 gene. Interestingly, silencer elements also mapped to the 5′ flanking region; however, these elements functioned in both pro-B cells and mature B cells. Further investigation will be necessary to evaluate the importance of negative regulation in mature B cells.

Here we present data to show that in pro-B cells, the Id1 gene is activated by a pro-B-cell-specific enhancer element located downstream of the structural gene. A 6-kb 3′ flanking sequence was found to be both necessary and sufficient for regulating the proper expression of the Id1 gene in pro-B cells, as demonstrated by the analyses of pro-B-cell lines stably transfected with various Id1 minigene constructs. Subsequent studies using transient transfection assays have led to the identification of a 74-bp pro-B-cell-specific enhancer. This enhancer satisfies the criteria for a classical enhancer in that it activates transcription from a distance when it is placed in either orientation and at either end of the gene. The enhancer also acts on a heterologous promoter.

EMSAs revealed a pro-B-cell-specific complex binding to the sequence TTGCATCA, called PBE, located in the 74-bp enhancer. Mutations in the sequence led to a great, but not complete, reduction of enhancer activity in pro-B cells. Whether this sequence is the sole site that mediates the enhancer activity remains to be determined.

The pro-B-cell-specific complex appears to be a novel complex, judging by its DNA binding specificity and its cell type distribution. Although the sequence shows a limited similarity to the AP-1 site, we have demonstrated that AP-1 sequences cannot eliminate the binding. In addition, no AP-1 binding activity has been described as having a pro-B-cell-specific pattern. The PBE sequence also partially overlaps with the W site, GTTGCATC, which is found in the enhancer of the major histocompatibility complex class II gene and forms a B-cell-specific complex, designated NF-W1, and a ubiquitous complex, called NF-W2 (8). The pro-B-cell-specific PBE complex is obviously not NF-W2. We have also provided evidence suggesting that the complex is distinct from NF-W1. However, since the PBE site and the W site overlap, it would be interesting to determine whether these PBE and W complexes have any common structural component and whether different heteromeric complexes are formed at different stages of B-cell development. By binding to the PBE or W site in the enhancer, different complexes may exert different actions.

The two pro-B-cell lines, LyD9 and Ba/F3, display the pro-B-cell-specific complex, which correlates with the high expression level of the Id1 gene in these cells. Thus, one might propose that the protein(s) binding to the PBE sequence is a transcription activator(s) for the expression of the Id1 gene in pro-B cells. During B-cell differentiation, the pro-B-cell-specific complex disappears and, consequently, Id1 expression diminishes. How might the specificity be governed? The specificity of this complex may be mediated through a pro-B-cell-specific synthesis of the proposed transcription factor(s) or a posttranslational modification of the factor(s) which prevents DNA binding at later stages of B-cell development. Such posttranslational modification can be achieved by phosphorylation or dephosphorylation of the DNA binding domain of the factor(s) in response to signals for B-cell differentiation or by association of an inhibitory protein, which is produced at later stages in B-cell development, with the factor(s) (2, 14). Investigation of the mechanisms awaits further characterization of the pro-B-cell-specific complex and the cloning of the cDNA(s) encoding the binding protein(s). Understanding the regulation of the Id1 gene in B cells will certainly shed light on mechanisms controlling B-cell development.

Interestingly, the Id1 gene is not only expressed in pro-B cells but also in other immature cells, such as myoblasts, embryonic carcinoma cells, and undifferentiated erythroid cells, but the pro-B-cell-specific complex is not found in these cells. There must be additional mechanisms that control Id1 expression in these other cells, or else different proteins which exhibit different gel shift patterns are bound to the same enhancer sequence. In fact, we have found that the substitution mutation in the PBE sequence of construct pfLUC/B1s causes at least an 80% reduction of the enhancer activity in C2C12 myoblasts and F9 embryonic carcinoma cells (data not shown), which suggests that the same sequence is involved in the activation of the Id1 gene in these cells through the action of a different protein complex. Further investigation is necessary to fully understand the regulation of the Id1 gene in these cell types.

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