

Regulation of the Pro-B-Cell-Specific Enhancer of the *Id1* Gene Involves the C/EBP Family of Proteins

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Received 18 September 1996/Returned for modification 22 October 1996/Accepted 7 November 1996

The *Id1* protein acts as a negative regulator in early-B-cell differentiation by antagonizing the function of the basic helix-loop-helix transcription factors. Expression of the *Id1* gene during B-cell development is governed at the transcriptional level primarily by a pro-B-cell-specific enhancer (PBE) located 3 kb downstream of the gene. We report here the identification of CAAT/enhancer binding protein β (C/EBP β) as a component of the two major PBE-binding complexes (PBEC1 and PBEC2) found in pro-B cells by gel mobility shift assays. Formation of the PBECs is abolished when a classic C/EBP binding site is used as a competitor, and binding complexes similar to the PBECs are formed when the classic C/EBP site is used as a probe. We show that CHOP, a negative regulator of C/EBPs, specifically inhibits PBE binding in vitro and its enhancer activity in vivo. In pro-B cells, C/EBP β binds to the PBE site not as apparent homodimers but possibly in association with at least one other polypeptide, which might determine the pro-B-cell-specific expression of the *Id1* gene. Although isoforms of C/EBP β are expressed in various B cells, they bind to DNA only in LyD9 and Ba/F3 pro-B cells. We show that CHOP is expressed in 70Z/3 and WEHI-231 cells. We also demonstrate that CHOP is associated with C/EBP β in WEHI-231 cells, which may provide an additional mechanism to control the function of C/EBP β and the expression of the *Id1* gene.

Id1 is a member of the gene family that encodes negative regulators for the basic helix-loop-helix (bHLH) transcription factors (7, 36). Id proteins, which have the HLH domain but no basic region, dimerize with the E protein subclass of bHLH factors (E2A, E2-2, and HEB) and render them inactive for DNA binding or dimerization with other bHLH proteins. As a result, these transcription factors cannot activate downstream target genes. The opposing functions of the bHLH and Id proteins dictate the cellular programs for differentiation and proliferation of various cell types, i.e., the bHLH factors promote differentiation and suppress proliferation while Id proteins block the former and stimulate the latter (5, 18, 20, 23, 25, 33, 35).

The development of B lymphocytes can be divided into pro-B, pre-B, and mature B stages according to the rearrangement and expression of immunoglobulin (Ig) genes (17, 29). Pro-B cells are the earliest identified stage, during which the Ig genes are in the germ line configuration. When these cells undergo rearrangement and expression of the Ig heavy-chain locus, they become pre-B cells. Mature B cells have both the heavy-chain and light-chain loci rearranged and express surface IgM. The *Id1* gene is thought to be expressed only in pro-B cells but not in later stages, while E2A gene products are present in all stages (19, 27, 36, 43). Constitutive expression of the *Id1* gene in transgenic mice blocks the development of B cells at the pro-B stage, a phenotype similar to that caused by the null mutations of the *E2A* gene (3, 35, 45). This and several lines of evidence suggest a counter functional relationship between E proteins and Id in regulating B-cell development. In pro-B cells, Id proteins keep the existing E proteins inactive, while in subsequent stages, *Id* expression is turned off to allow

E proteins to stimulate the expression of downstream targets required for the differentiation of B cells. Therefore, expression of *Id* genes must be precisely regulated in order for the differentiation to proceed.

To elucidate the mechanisms regulating *Id1* gene expression, we have previously shown that the *Id1* gene is controlled at the transcriptional level by an enhancer element located approximately 3 kb downstream of the structural gene (31). This element can mediate the transcriptional activation of the luciferase reporter constructs driven by the promoter of the *Id1* gene or by the minimal promoter of the *c-fos* gene only in LyD9 and Ba/F3 pro-B-cell lines but not in PD31 pre-B and WEHI-231 mature-B-cell lines. (Although it is debatable whether LyD9 and Ba/F3 represent pro-B cells or earlier progenitors in hematopoietic development, for the sake of simplicity, we will refer to them as pro-B-cell lines.) This enhancer element was thus named PBE for its specific activity at the pro-B-cell stage. Specific PBE-binding complexes were also detected in nuclear extracts from pro-B cell lines, and they are referred to as PBE complexes (PBECs). To further understand B-cell stage-specific regulation of the *Id1* gene, we screened a pro-B-cell cDNA expression library using concatemeric PBE sites as a probe and identified CAAT/enhancer binding protein β (C/EBP β) as a positive clone. We subsequently provided several lines of in vitro and in vivo evidence to demonstrate that C/EBP β is a component of PBECs not as homodimers but as heteromers with an unknown protein in pro-B cells. In WEHI-231 mature B cells, C/EBP β is bound to CHOP, an inhibitor in the C/EBP family, and is thus inactivated. We suggest that the C/EBP family of proteins, by forming pro-B-cell-specific active complexes or mature-B-cell-specific inactive complexes, may play important roles in the regulation of the *Id1* gene during B-cell development.

MATERIALS AND METHODS

Cell lines, plasmids, and antibodies. Mouse B-cell lines (LyD9, Ba/F3, PD31, 70Z/3, and WEHI-231) were grown in RPMI medium supplemented with 10% fetal bovine serum and 50 μ M β -mercaptoethanol. The medium for LyD9 and

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Ba/F3 was also supplemented with 10% WEHI-3-conditioned medium as a source of interleukin-3. The luciferase reporter constructs were described previously (31). Constructs overexpressing C/EBP β and CHOP in pcDNA 1 (Invitrogen, San Diego, Calif.) were gifts from David Ron (30). C19 and Δ 198 antibodies against C/EBP β were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). The antiserum specific to the 31-kDa form (LAP) of C/EBP β (NF66) and the monoclonal antibody to CHOP (37-9C8) were also gifts from David Ron (6, 30).

Library construction and screening. A λ gt11 library was generated from random-primed cDNAs of poly(A)⁺ RNA isolated from LyD9 pro-B cells by using the Stratagene (La Jolla, Calif.) cDNA Synthesis and Vector kits. A total of 9×10^5 plaques were screened with a 5-concatemered PBE probe according to the protocol described elsewhere (34, 40). The wild-type (WT) PBE sequence TCGAGGGTTGCATCAGGGCGTCA represents one unit of the concatemered probe sequence. The PBE site is underlined and only the 5'-3' top strand is shown. The positive clone was purified, and the phage DNA was prepared. The insert was excised by digestion with *Eco*RI and was subcloned into the pBlue-script II KS⁺ (Stratagene) plasmid for sequencing.

EMSA, competition study, and supershift analysis. The electrophoretic mobility shift assay (EMSA) and competition studies were performed as described elsewhere (31). Typically, 10^4 cpm of the 97-bp PBE-containing probe and 5 μ g of crude nuclear extract were used in a binding reaction followed by analyses using 6% nondenaturing polyacrylamide gels and the Tris-glycine buffer unless otherwise indicated. For binding reactions with nuclear extract from COS-1 cells overexpressing C/EBP β , 50 ng of the extract mixed with 5 μ g of nontransfected COS-1 nuclear extract was used in each reaction. For supershift assays, 1 μ g of the C19 or Δ 198 antibody was added to the binding reaction mixture and incubated for 5 min before gel electrophoresis. In the EMSA involving CHOP, bacterially expressed glutathione *S*-transferase (GST)-CHOP or GST-CHOP LZ⁻ (gifts from David Ron) in the amounts equivalent to those used to disrupt the binding of C/EBP α or C/EBP β homodimers was incubated for 15 min with 5 μ g of LyD9 crude nuclear extract prior to the binding reaction as previously described (30). The acute-phase response element (APRE)-M6 sequence, which contains a classic C/EBP binding site with the mutation (M6) that eliminated NF- κ B binding to the APRE, was synthesized according to the published sequence (8). The sequence of oligonucleotide competitor (5'-3' top strand) of the mutated PBE is TCGAGCCGTCGATCACCCATCGTCA. The WT sequence is the same as that of the WT PBE oligonucleotide shown above. The PBE site is underlined and the mutated nucleotide is lowercase. Complexes bound to the APRE-M6 probe were resolved on 6% acrylamide gels in 0.5 \times Tris-borate-EDTA (TBE) buffer.

Transfection and assay of luciferase activity. Approximately 5×10^6 LyD9 cells were transfected with 5 μ g of reporter plasmid, 20 μ g of CHOP, CHOP LZ⁻, or carrier plasmid, and 1.5 μ g of cytomegalovirus (CMV)-*lacZ* reporter as an internal control by the DEAE-dextran method (2). Cells were collected and assayed for luciferase and β -galactosidase activities after 2 days by using luciferase assay substrate (Promega, Madison, Wis.) and the Galacto-light Plus kit (Tropix, Bedford, Mass.) respectively. Raw data of luciferase light units were first normalized with β -galactosidase activities. The relative activities were then calculated by dividing the normalized activities with that of the pFLUC construct, which represents basal activity. The data presented are from three separate transfection experiments.

Western blot (immunoblot) analysis and coimmunoprecipitation. Fifty micrograms of each nuclear extract was loaded on sodium dodecyl sulfate (SDS)-12% polyacrylamide gels, and proteins were transferred to nitrocellulose membranes after electrophoresis. Western blots were performed with a 1:10,000 dilution of the C19 anti-C/EBP β antibody or a 1:5,000 dilution of anti-CHOP antibody. The immune complexes were detected with a 1:2,500 dilution of the secondary horseradish peroxidase-conjugated goat-anti-rabbit or anti-mouse IgG (Promega) and the ECL detection system (Amersham). For C/EBP β -CHOP coimmunoprecipitation, 4×10^7 LyD9 or WEHI-231 cells were lysed in 1.5 ml of radioimmunoprecipitation assay (RIPA) buffer by passing through a 21-gauge needle four to five times. Five micrograms of the C19 antibody was added to the lysate after preclearing according to the standard protocol (2). To disrupt CHOP-C/EBP β complexes, 4×10^7 WEHI-231 cells were lysed in 150 μ l of RIPA buffer with 1% SDS and heated at 100°C for 5 min. The lysate was then brought up to 1.5 ml by addition of RIPA buffer without SDS followed by immunoprecipitation with the C19 antibody. The precipitates were resuspended in 60 μ l of 2 \times sample buffer and heated at 100°C for 5 min. One-third of each sample was separated on an SDS-12% polyacrylamide gel and Western blotted with the anti-CHOP antibody.

RESULTS

C/EBP β binds to PBE and is expressed in B cells. To identify the protein(s) that interacts with the PBE site, we constructed a λ gt11 expression library from LyD9 pro-B-cell cDNAs and screened the library with a concatemer probe consisting of five copies of the PBE site. After 900,000 plaques had been screened, one positive clone was obtained. Sequence analysis of this clone revealed that it contained the cDNA

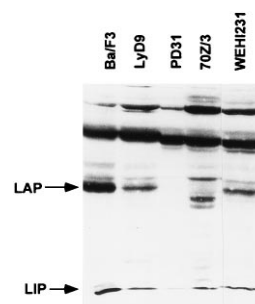


FIG. 1. Expression of C/EBP β in mouse B-cell lines; Western blot analysis of nuclear extracts from Ba/F3 and LyD9 pro-B cells, PD31 and 70Z/3 pre-B cells, and WEHI-231 mature B cells loaded as indicated. The C19 antibody directed against the carboxy terminus of C/EBP β was used. Both isoforms (LAP and LIP) were detected by this antibody as indicated.

encoding C/EBP β . To determine the pattern of C/EBP β expression in B cells, Western blot analysis with an anti-C/EBP β antibody (C19) was performed. By alternative translation initiation, the mouse C/EBP β transcript encodes three polypeptides with calculated molecular masses of 31, 29, or 16 kDa (10, 16). The two larger forms contain the transcriptional activation domain and are also termed liver-enriched transcriptional activator proteins (LAPs) (16). The smallest form lacks the activation domain and has a dominant negative effect on LAPs. It is therefore called liver-enriched transcriptional inhibitory protein (LIP) (16). Both the LAP (31-kDa) and LIP forms of C/EBP β are detected in Ba/F3 and LyD9 pro-B cells but not in PD31 pre-B cells (Fig. 1). In 70Z/3 pre-B cells, the 31-kDa LAP is not present, but an antibody-reacting polypeptide that could correspond to the 29-kDa form of LAP is detected along with LIP. Interestingly, both LAP and LIP are found in WEHI-231 mature B cells, even though the PBECs are not detected in EMSAs. This raises the possibility that C/EBP β may require a pro-B-cell-specific binding partner to form PBECs in pro-B cells or that a negative regulator may prevent C/EBP β from binding to PBE in 70Z/3 and WEHI-231 cells.

PBEC formation can be inhibited by a classic C/EBP binding site. We have previously described a pro-B-cell-specific binding complex, now called PBEC1, observed in EMSAs with a 97-bp PBE probe and 4% nondenaturing gels (31). By increasing the resolution of the polyacrylamide gels (6%), we subsequently discovered another faster-migrating pro-B-cell-specific complex and named it PBEC2 (Fig. 2). Since C/EBP β was shown to bind to PBE by Southwestern screening of a λ gt11 library and since the PBE site was not previously recognized by computer analysis as a C/EBP binding site (31), we attempted to verify whether C/EBP β is a component of the PBECs found in pro-B cells. We first asked whether the PBECs share any DNA binding specificity with the C/EBPs. We performed a competition assay using oligonucleotides containing a classic C/EBP binding site (APRE-M6) as competitors in EMSA (8). The 97-bp probe bearing the PBE site was incubated with the LyD9 pro-B-cell nuclear extract in the absence or presence of the competitors (Fig. 2). The APRE-M6 oligonucleotides competed off PBEC1 and PBEC2 nearly as efficiently as the WT PBE oligonucleotides, while a pair of PBE mutant oligonucleotides did not affect PBE binding at the same concentrations. We thus conclude that components of PBECs can also bind to the classic C/EBP binding site.

CHOP inhibits PBEC formation. To provide additional evidence that PBECs may contain C/EBP β , we tested if the formation of PBECs can be abolished by CHOP, an inhibitor

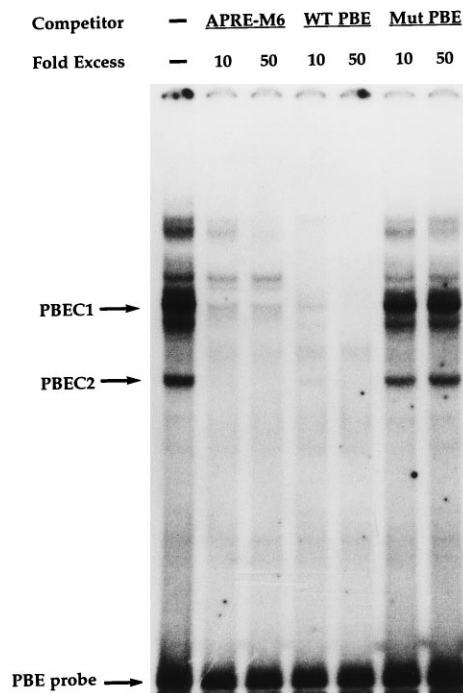


FIG. 2. Competition of pro-B-cell-specific binding by a classic C/EBP site. An EMSA was carried out with LyD9 nuclear extract and the PBE probe. Unlabeled oligonucleotide competitors were added to the binding reaction mixtures in excess of the probe as indicated. APRE-M6 is an oligonucleotide containing the classic C/EBP binding site, WT PBE carries the minimal PBE sequence TTGCATCA, and Mut PBE contains a single-base pair mutation of the PBE sequence, GTGCATCA. Complexes were resolved on a 6% acrylamide nondenaturing gel. The migrations of the pro-B-specific complexes are also indicated.

of C/EBP α and - β (30). Bacterially expressed GST-CHOP, in the amounts comparable to those used to inhibit DNA binding by C/EBP β homodimers, was added to the binding reaction mixtures in the EMSA (Fig. 3). Addition of CHOP at the concentration of 50 ng per reaction mixture begins to inhibit PBE binding activity and at 500 ng per reaction mixture completely abolishes both PBEC1 and PBEC2. In contrast, the CHOP LZ⁻ protein, which lacks the leucine zipper dimerization domain, cannot inhibit binding even at 500 ng per reaction mixture. These results further suggest that PBECs consist of one or more members of C/EBP family, which can dimerize with CHOP through the leucine zipper domain.

CHOP inhibits the enhancer activity of PBE. We then showed that the pro-B-cell-specific enhancer activity of PBE can be inhibited by CHOP in vivo. Cotransfection of the CHOP or CHOP LZ⁻ expression plasmid with the luciferase reporter gene controlled by PBE (pFLUC/B1) was performed (Fig. 4). Compared to the expression of the reporter gene without the enhancer (pFLUC), the PBE-containing sequence (pFLUC/B1) activates the reporter by about 22-fold, while mutation at the PBE site (pFLUC/B1s) abolishes 60% of this activity. CHOP but not CHOP LZ⁻ inhibits 90% of the PBE activity. Apparently, the inhibitory effect by CHOP is greater than the mutation at the PBE site itself. This is probably due to the presence of other potential C/EBP binding sites in the reporter plasmid. For example, two such sites are found 5 bp downstream and 50 bp upstream from the PBE. The presence of these putative C/EBP sites may explain why CHOP could further inhibit the remaining activity of the mutated PBE construct (pFLUC/B1s). However, the inhibition by CHOP is spe-

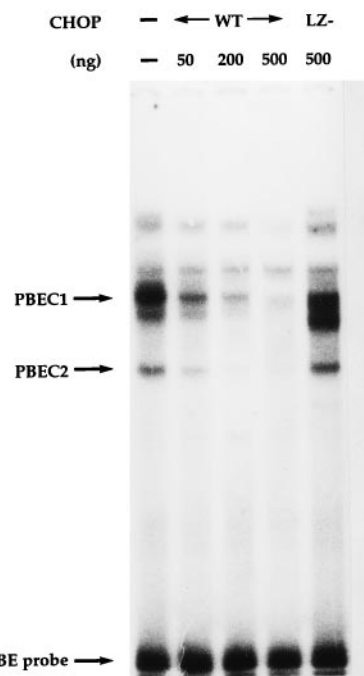


FIG. 3. CHOP inhibits the pro-B-cell-specific binding. An EMSA was performed with LyD9 nuclear extract and the PBE probe as described in the legend to Fig. 2. GST-CHOP or GST-CHOP-LZ⁻ fusion proteins expressed in bacteria were added to the binding reaction mixtures without the probe and incubated for 15 min before the binding reactions were started by addition of the probe. Each reaction mixture contained 5 μ g of the LyD9 nuclear extract.

cific to the PBE reporter because expression of the CMV-*lacZ* reporter construct used as an internal control was not affected by the presence of CHOP (data not shown). Taken together, these in vitro binding and in vivo transactivation data support the idea that members of the C/EBP family are involved in the binding of the PBE enhancer and the regulation of the *Id1* gene.

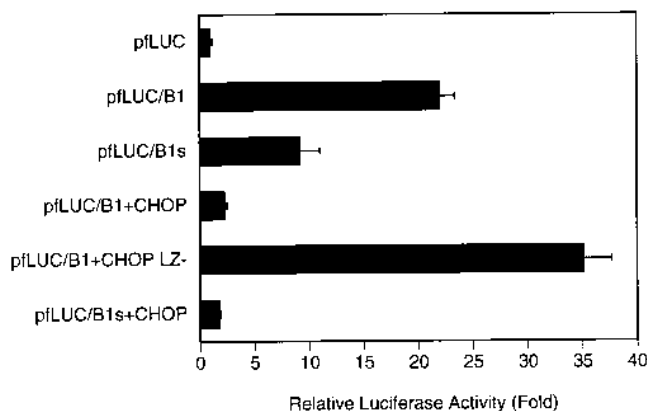


FIG. 4. CHOP inhibits pro-B-cell-specific *Id1* enhancer activity. LyD9 cells were transiently transfected with the luciferase reporter constructs as indicated. pFLUC is a reporter controlled by the minimal promoter of the *c-fos* gene. B1 and B1s, reporters in which a 500-bp WT and mutant PBE-containing enhancer were cloned in pFLUC, respectively. CHOP and CHOP-LZ⁻ expression plasmids were each cotransfected with pFLUC/B1 or pFLUC/B1s as indicated. Data from three independent transfections are presented as fold of activation (averaged) relative to pFLUC activity.

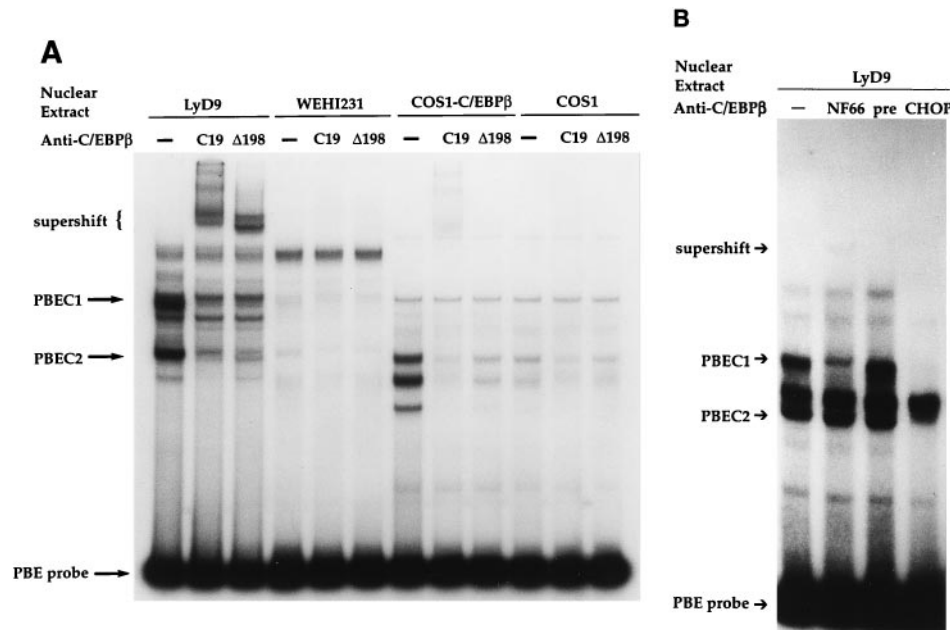


FIG. 5. The PBECs are not C/EBP β homodimers. (A) An EMSA was performed with the PBE probe and nuclear extracts from LyD9, WEHI-231, C/EBP β -expressing COS-1, and COS-1 cells. The different antibodies to C/EBP β added to the binding reaction mixture are indicated at the top of the lanes. Supershift, new complexes formed after antibody addition. (B) A similar EMSA was performed with LyD9 nuclear extract. An antiserum (NF66) specific to the LAP isoform of C/EBP β was used. A preimmune serum was added to one binding reaction mixture as a control. A 100-ng amount of GST-CHOP was also added to another binding reaction mixture to identify C/EBP-related binding complexes. The binding reactions were analyzed using a 4% nondenaturing polyacrylamide gel.

PBECs contain C/EBP β -, but the binding complexes are distinct from the C/EBP β homodimer complexes. To determine whether C/EBP β is a component of the PBECs, we performed an EMSA in the presence of antibodies against C/EBP β . Both PBEC1 and PBEC2 in LyD9 cells were supershifted by two different antibodies against C/EBP β , C19, and Δ 198 (Fig. 5A). Apart from PBEC1 and PBEC2, LyD9 cells also display several minor complexes that cannot be supershifted by the anti-C/EBP β antibodies and are present in varying quantities in non-pro-B cells such as WEHI-231 cells (Fig. 5A). Nuclear extract from WEHI-231 mature B cells did not show either PBEC1 or PBEC2; therefore, no supershifted complex was observed when the anti-C/EBP β antibodies were added to the binding reaction mixtures. Since C/EBP β proteins are known to bind to DNA as homodimers (16), we asked whether the PBECs and the C/EBP β binding complexes comigrate in gel electrophoresis. C/EBP β was overexpressed in COS-1 cells, and nuclear extract from these cells was prepared. In EMSAs, three PBE binding complexes were detected with this extract and could all be supershifted by the anti-C/EBP β antibodies. These three complexes possibly represent the homo- and heterodimers of the 31- and 16-kDa forms of the C/EBP β alternative translation products called LAP and LIP (16). The fastest-migrating complex would be to the LIP-LIP homodimer followed by the LAP-LIP heterodimer and the LAP/LAP homodimer. However, none of these complexes comigrates with either PBEC1 or PBEC2. Although PBEC2 and the potential LAP-LAP homodimer appeared to migrate similarly in this gel, we found them to be distinct upon prolonged electrophoresis (data not shown). Currently, we cannot rule out the possibility that different posttranslational modifications in LyD9 and COS-1 cells which would result in different migrating properties of C/EBP β homodimers in EMSA may have occurred. However, our data strongly suggest that the PBECs are unlikely the simple homodimers of C/EBP β .

Rather, they are probably heterodimers associated with a protein(s) that has not yet been identified and that may or may not be a member of the C/EBP family. Supershift experiments have ruled out C/EBP α or c-Fos as a dimerization partner of C/EBP β in the PBECs (data not shown).

A minor band migrating faster than PBEC2 could also be supershifted by the anti-C/EBP β antibodies. It probably represents another C/EBP β complex either formed between its isoforms (e.g., LAP-LIP) or associated with other members of the C/EBP family (e.g., Ig/EBP).

What is the difference between PBEC1 and PBEC2? One likely explanation is that PBEC1 contains the 31-kDa LAP, while PBEC2 has the 16-kDa LIP. The supporting evidence came from an EMSA in the presence of a LAP-specific antibody, NF66 (Fig. 5B). Only PBEC1 but not PBEC2 was supershifted by the addition of this antibody. Incidentally, both the 31- and 16-kDa forms of C/EBP β are expressed in LyD9 cells (Fig. 1). However, it is not clear whether PBEC1 and PBEC2 both activate transcription through PBE. This experiment also helps to argue that the PBECs may not contain the C/EBP β homodimers. For example, the lack of LAP in PBEC2 would rule out the possibility that PBEC2 is similar to the LAP-LAP homodimer in the C/EBP β -transfected COS-1 cells (Fig. 5A). The EMSA shown in Fig. 5B also displays a binding complex, which cannot be eliminated by CHOP and is present in only some of the preparations of nuclear extracts.

C/EBP β in pro-B but not in mature B cells binds to the classic C/EBP binding site. Since C/EBP β is also expressed in WEHI-231 mature B cells (Fig. 1), we were intrigued by the fact that it did not form complexes with PBE. One possibility is that C/EBP β homodimers cannot bind to the PBE site efficiently and can do so only when overexpressed in COS-1 cells. We then asked whether C/EBP β in WEHI-231 can bind to the classic C/EBP binding site. An EMSA was performed with a pair of the APRE-M6 oligonucleotides as a probe (Fig. 6). We

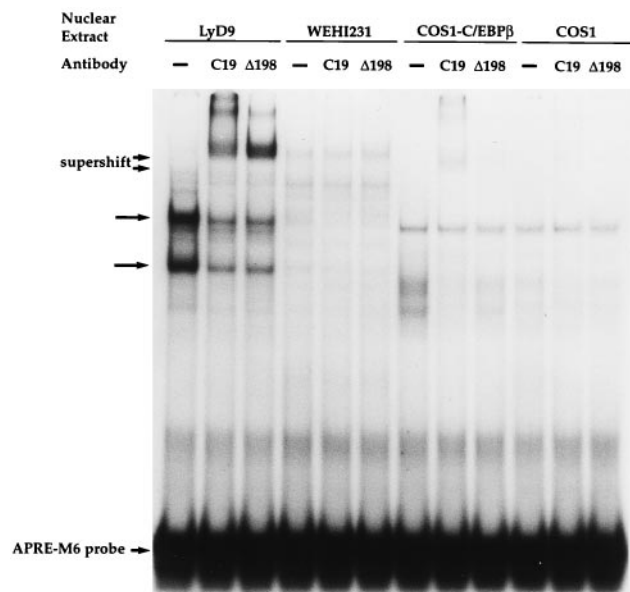


FIG. 6. C/EBP β in LyD9 but not in WEHI-231 cells binds to the classic C/EBP site. The APRE-M6 probe and nuclear extracts from LyD9, WEHI-231, COS-1-expressing C/EBP β , and COS-1 cells were employed in an EMSA. Antibodies to C/EBP β were added to the binding reaction mixtures as indicated. Pro-B-cell-specific complexes and supershift complexes associated with the antibodies are indicated by arrows.

observed two binding complexes from the LyD9 nuclear extract that were supershifted by the two anti-C/EBP β antibodies. These complexes are probably equivalent to the PBECs found with the PBE probe. Again, none of these complexes comigrated with the C/EBP β dimer complexes in the C/EBP β -expressing COS-1 extract. In agreement with the finding with PBE as a probe, the WEHI-231 extract did not display any obvious binding complex that reacts with the anti-C/EBP β antibodies. These results led us to conclude that C/EBP β in WEHI-231 mature B cells is unable to bind to DNA even as a homodimer.

CHOP is expressed and associated with C/EBP β in WEHI-231 cells. One possible factor that can prevent C/EBP β from binding to DNA is CHOP (30). Therefore, we tested whether CHOP was expressed in any of the B-cell lines. Western blot analysis revealed that CHOP is expressed in the 70Z/3 pre-B and WEHI-231 mature-B-cell lines but not in Ba/F3, LyD9,

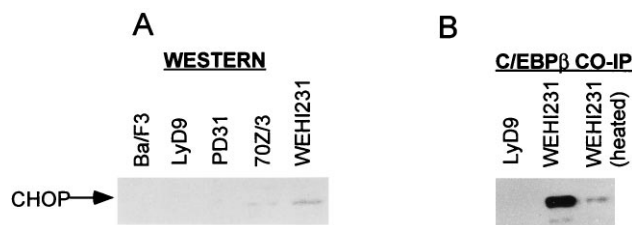


FIG. 7. CHOP is expressed and associated with C/EBP β in mature B cells. (A) CHOP is expressed in 70Z/3 pre-B and WEHI-231 mature B cells but not in Ba/F3, LyD9, and PD31 cells. A Western blot analysis of nuclear extracts from various B-cell lines was performed with a monoclonal antibody to CHOP. (B) CHOP in WEHI-231 mature B cells is associated with C/EBP β . Extracts from LyD9 and WEHI-231 cells were immunoprecipitated with the C19 anti-C/EBP β antiserum. The immune complex was resolved on an SDS-polyacrylamide gel electrophoresis gel, and CHOP was detected by Western blotting. In the lane marked heated, another sample of WEHI-231 extract was heated at 100°C for 5 min in 1% SDS prior to immunoprecipitation.

and PD31 cell lines (Fig. 7A). The presence of CHOP in 70Z/3 and WEHI-231 cells may explain why C/EBP β can bind to DNA only in LyD9 and Ba/F3 cells even though 70Z/3 and WEHI-231 cells have equivalent levels of C/EBP β (Fig. 1). C/EBP β in 70Z/3 and WEHI-231 cells may be sequestered by CHOP and cannot homodimerize to bind to DNA.

To test this hypothesis, we then investigated whether CHOP and C/EBP β in WEHI-231 cells are physically associated with each other. The C19 antibody to C/EBP β was first used to immunoprecipitate extracts from WEHI-231 as well as LyD9 cells as a control. The immune complexes were then dissociated and analyzed by electrophoresis on an SDS-polyacrylamide gel electrophoresis gel. Subsequent Western blot analysis with an antibody against CHOP revealed that CHOP in the WEHI-231 extract was brought down by the antibodies against C/EBP β (Fig. 7B), indicating an interaction between C/EBP β and CHOP. To disrupt the interaction, the WEHI-231 cell extract was boiled for 5 min in RIPA buffer containing 0.1 M NaCl-1% SDS. After this treatment, a significantly smaller amount of CHOP was coimmunoprecipitated with C/EBP β . CHOP is not expressed in LyD9 cells; therefore, it is not detected in the immunoprecipitated complex. We thus conclude that C/EBP β in WEHI-231 cells is associated with CHOP, which prevents C/EBP β from binding to either the classic C/EBP site or the PBE site.

DISCUSSION

The C/EBP family and its role in B-cell development. We have found previously that the PBE element located 3-kb downstream of the *Id1* gene is central to regulation of the *Id1* gene in pro-B-cell lines (31). Here, we report the identification of C/EBP β as one of the protein components that forms the pro-B-cell-specific PBECs, PBEC1 and PBEC2. There are several minor binding complexes in the pro-B-cell lines, but they are also present in WEHI-231 cells in quantities that vary from preparation to preparation. Some of these complexes cannot be competed off by the classic C/EBP oligonucleotides, while others cannot be inhibited by CHOP. Currently, we have not determined the identities and the functional significance of these minor complexes. However, the functional relevance of the C/EBP β containing PBEC1 and PBEC2 is emphasized by the fact that they represent the major PBEC existing only in *Id1*-expressing pro-B cells.

C/EBP β (IL-6DBP and NF-IL6) belongs to a large protein family consisting of C/EBPs (1, 16, 26). These C/EBPs contain a conserved basic region and leucine zipper domain for DNA binding and dimerization (41). Members of this family act as either transcription activators or repressors in cell proliferation and differentiation (9, 39). They also mediate signal transduction (11, 37). Although the gene encoding C/EBP β does not contain any intron, alternative translation initiation of the C/EBP β transcript generates two major forms of the polypeptides, LAP and LIP (16). Because LIP does not possess the activation domain of LAP, it can bind to DNA but activates transcription poorly. Therefore, LIP may act as a dominant negative inhibitor to other C/EBPs under certain circumstances.

Ig/EBP and C/EBP β have been shown previously to be members of the C/EBP family that are expressed in the B-cell lineage (14). Ig/EBP is present throughout all stages of B-cell development, while C/EBP β is turned on later at mature B and plasma stages. We have shown here that C/EBP β is also expressed in two pro-B-cell lines, LyD9 and Ba/F3 (Fig. 1). While Ig/EBP is considered a general dominant negative inhibitor (12), the role of C/EBP β may be complicated by the presence

of LAP and LIP isoforms, which act as an activator and a repressor, respectively. The classic C/EBP sites have been shown in cell culture to be important for the transcription of Ig V_H transcripts (13), as well as for the expression of the Ig γ (24, 44) and ϵ (15) sterile transcripts involved in class switching at later stages of B-cell development. C/EBP β is also demonstrated in cell culture systems to activate interleukin-6 (IL-6) expression (1) and to participate in the IL-6 signaling pathway (11). However, the phenotype of C/EBP β -deficient mice suggests that C/EBP β acts as a negative factor in the lymphoid lineage (32). These mutant mice display an expansion of the B-cell compartment filled with B cells expressing surface IgG1, suggesting that class switching has taken place in the absence of C/EBP β . They also have high rather than low levels of IL-6 in serum, as one would predict based on the data from cell culture studies. Although our results have shown that C/EBP β may be involved in the activation of *Id1* gene expression in pro-B cells, lack of C/EBP β in mutant mice does not cause any defect in early-B-cell development. This is not surprising, because if reduction of *Id1* expression occurs, it may be compensated by the presence of other Id proteins.

One exceptional member of the C/EBP family is CHOP, which has a nonconserved basic domain compared with other family members (30). CHOP can dimerize with C/EBP α or β and prevent them from binding to classic C/EBP binding sites. We have shown that CHOP is expressed and bound to C/EBP β in WEHI-231 mature B cells; however, CHOP is not present in the pro-B-cell lines. Interestingly, expression of CHOP has recently been shown to be induced by agents that cause endoplasmic reticulum (ER) stress by adversely affecting the function of the ER (28, 42). It would be interesting to determine if the increased burden of the ER to translocate and secrete Igs in mature and plasma cells can also induce CHOP. The CHOP-C/EBP β heterodimers are also shown to be able to bind to DNA sequences distinct from classical C/EBP sites (38). However, the physiological target genes controlled by the heterodimers have not been identified.

Regulation of the *Id1* gene by C/EBPs. Regulation of *Id1* gene expression is critical for early-B-cell development (21, 27, 35, 43). *Id1* is expressed at high levels in LyD9 and Ba/F3 pro-B-cell lines but is dramatically down-regulated in pre-B, mature-B, and plasma cell lines (36, 43). One of the major regulatory mechanisms is thought to involve the pro-B-cell-specific transcriptional activation mediated by the PBE element (31). PBE forms two pro-B-cell-specific complexes in EMSA (PBEC1 and PBEC2), which are likely the heteromers between the LAP or LIP form of C/EBP β and at least one unknown protein. Based on the evidence presented here, we propose a mechanism for the regulation of the *Id1* gene in B-cell development. In pro-B cells, C/EBP β forms complexes with the unknown protein, termed X, to activate *Id1* expression. However, it is not clear whether both PBEC1 and PBEC2 can activate transcription. This will depend on whether the activation domain in LAP is required for transcriptional activation or whether protein X can stimulate transcription on its own. It also remains to be determined whether protein X is specifically expressed in pro-B cells and whether it can bind to PBE as homomers. Cloning the cDNA encoding protein X will greatly facilitate the investigation. Noticeably, a very small amount of complex that may correspond to the LAP-LIP heterodimers is detected in LyD9 cells, which may imply that most C/EBP β proteins are bound to protein X or that C/EBP β does not bind to DNA efficiently as homodimers. In either case, the abundance of the heteromers between C/EBP β and protein X stresses their importance in regulating *Id1* expression.

In addition to controlling the expression or activity of pro-

tein X, the availability of C/EBP β may also be able to regulate the function of PBE. For example, in some pre-B cells in which C/EBP β is not expressed, PBECs cannot form even if protein X is present. In 70Z/3 pre-B and WEHI-231 mature B cells, despite the expression of C/EBP β , the presence of CHOP prevents C/EBP β from dimerizing with other proteins and even binding to the C/EBP sites as homodimers. So far, this model explains why *Id1* is expressed in pro-B cells but not in pre-B and WEHI-231 mature B cells. In late mature B and plasma cells, however, C/EBP β is detected in gel shift complexes bound to its classic binding sites (13). However, *Id1* is not expressed in these cells (43). The possible reasons for this are severalfold. First, the *Id1* gene could be activated only by the heterodimers between protein X and C/EBP β but not by C/EBP β homodimers. If so, expression of protein X would have to be shut off or the protein would have to be inactivated in mature B and plasma cells. Second, the level of intrinsic transactivating ability of the C/EBP β -containing complexes found in these mature B cells might not be high enough to stimulate *Id1* expression (14). Finally, the presence of *trans*-dominant negative inhibitors such as Ig/EBP and LIP might compete with the full-length C/EBP β (LAP) to bind to DNA and thus inhibit transcriptional activation (12). These last hypotheses appear to be supported by the phenotypes of C/EBP β -deficient mice, which suggest that C/EBP β may play a negative role in mature B cells (32).

In summary, we present evidence here to describe the interaction of the PBE element with C/EBP β and its unidentified associate as well as the role of CHOP in regulating C/EBP β function in WEHI-231 mature B cells. Since the DNA binding potential and transcriptional activating potential of the C/EBP family are known to be regulated by the signal transduction pathways of various growth factors and cytokines (22), it will be of profound interest to determine the effects of C/EBP β on *Id1* expression upon the posttranslational modification of C/EBP β . Furthermore, the finding that *Id1* expression can be inhibited by CHOP may suggest a possibility that induction of CHOP during growth arrest may lead to reduction of the expression of the growth-stimulating *Id1* gene (4, 5, 25).

Although PBEC1 and PBEC2 are not found in other *Id1*-expressing cells, including myoblasts, embryonic carcinoma cells, and myeloid precursor cells, the PBE site is indeed found to act as an enhancer there (30a, 31). It would be interesting to determine if the C/EBP family of transcription factors and protein X play any role in regulating *Id1* expression in these cells.

ACKNOWLEDGMENTS

We are indebted to David Ron for numerous suggestions, valuable reagents, and critical reading of the manuscript. We thank Herbert Samuels and Harinder Singh for technical advice in library screening. We also thank Muktar Mahajan for critical reading of the manuscript.

S.S. is a recipient of a scholarship from the Thai government.

This work was supported by NIH grant AI33597 and the Lucille P. Markey Charitable Trust. X.-H.S. is a Cancer Research Institute Investigator and an Irma T. Hirsch Trust scholar.

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