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Cloning and Characterization of the cDNA Encoding a Novel Human Pre-B-Cell Colony-Enhancing Factor

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A novel gene coding for the pre-B-cell colony-enhancing factor (PBEF) has been isolated from a human peripheral blood lymphocyte cDNA library. The expression of this gene is induced by pokeweed mitogen and superinduced by cycloheximide. It is also induced in the T-lymphoblastoid cell line HUT 78 after phorbol ester (phorbol myristate acetate) treatment. The predominant mRNA for PBEF is approximately 2.4 kb long and codes for a 52-kDa secreted protein. The 3′ untranslated region of the mRNA has multiple TATT motifs, usually found in cytokine and oncogene messages. The PBEF gene is mainly transcribed in human bone marrow, liver tissue, and muscle. We have expressed PBEF in COS 7 and PA317 cells and have tested the biological activities of the conditioned medium as well as the antibody-purified protein in different in vitro assays. PBEF itself had no activity but synergized the pre-B-cell colony formation activity of stem cell factor and interleukin 7. In the presence of PBEF, the number of pre-B-cell colonies was increased by at least 70% above the amount stimulated by stem cell factor plus interleukin 7. No effect of PBEF was found with cells of myeloid or erythroid lineages. These data define PBEF as a novel cytokine which acts on early B-lineage precursor cells.

Bone marrow cells serve as the primary site for B-cell lymphogenesis by providing a microenvironment of stromal cells in which the progenitor B cells can differentiate (7, 8). This process is finely regulated by both positive and negative signals at each stage of activation, proliferation, and differentiation. Essential regulatory elements for both early and late events in B-cell development have been demonstrated to be derived from adherent fibroblastic stromal cells (8). These include interleukin 1 beta (IL-1β) (1), IL-4 (29), IL-5 (30), IL-6 (12), IL-11 (26), and low- and high-molecular-weight B-cell growth factors (21). In addition, the early steps of B-cell lymphogenesis are controlled by IL-7 (20), which is synergized by stem cell factor (SCF) (17).

However, there seems to be a need for additional factors for the earliest events in B-cell development. Lee et al. (14) showed that the majority of cells that proliferate in response to IL-7 are $B220^+$ cells. Recently it was demonstrated that pre-B-cell formation from $B220^+$ immunoglobulin-negative progenitor cells and expression of μ heavy chain of immunoglobulin are uniquely dependent on the presence of S17 stromal cells and cannot be stimulated with either IL-7, SCF, or both IL-7 and SCF (2). Primitive cells, defined as lineage negative/Sca-1 positive ($Lin\text{-}1^-$ $Sca\text{-}1^+$), do not respond to SCF plus IL-7, but in the presence of SCF plus IL-3 and stromal cells, $Lin\text{-}1^-$ $Sca\text{-}1^+$ cells generate $B220^+$ cells (16a). Detailed studies by Landreth et al. (9, 13) suggest that additional stromal-cell-derived but not-yet-characterized cytokines control the developmental step in which the maturation of immature B-cell precursors to an IL-7–SCF-responsive state occurs.

In this report, we demonstrate that the effect of SCF and IL-7 on murine pre-B-cell colony formation can be significantly enhanced by a novel cytokine-like molecule. This molecule is the product of a novel gene which is expressed in bone marrow stromal cells as well as in activated human lymphocytes. We have isolated and characterized the cDNA clone from activated lymphocytes and have termed this protein pre-B-cell colony-enhancing factor (PBEF).

MATERIALS AND METHODS

Reagents. Human peripheral blood enriched in mononuclear cells was bought from Hemacare, Sherman Oaks, Calif. Some cDNA libraries and multiple tissue Northern (RNA) blots and RNA from various human tissues were bought from Clontech Laboratories (Palo Alto, Calif.). Oligonucleotide primers and probes were synthesized on an Applied Biosystems (Foster City, Calif.) model 380A oligonucleotide synthesizer. Antibodies were raised at Antibodies Inc. (San Diego, Calif.). The plasmid DNA isolation kit was from Qiagen (Studio City, Calif.). Restriction enzymes were bought from Boehringer Mannheim (Indianapolis, Ind.) and GIBCO-BRL (Gaithersburg, Md.).

Isolation and induction of human peripheral blood lymphocytes. Peripheral blood lymphocytes were isolated from freshly prepared buffy coats on a Ficoll-Paque step gradient (Pharmacia, Uppsala, Sweden). Mononuclear cells present in the interphase were removed and washed with phosphate-buffered saline (PBS) three times and then suspended in RPMI 1640–10% fetal calf serum. About 5 million cells per ml were incubated with pokeweed mitogen (10 μg/ml; Sigma) for 15 h; incubation was followed by an additional incubation for 4 h in the presence of cycloheximide (10 μg/ml). Incubation was carried out at 37°C in 5% CO_2.

Construction of cDNA libraries. Total RNA was isolated from activated lymphocytes by a guanidium thiocyanate-CsCl technique (5). Polyadenylated RNA was selected by oligo(dT) chromatography, ethanol precipitated, and centrifuged. The final pellet was dissolved in water and kept in liquid nitrogen in aliquots.

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About 5 μg of poly(A)$^+$ RNA was used for cDNA library construction. After denaturation with methyl mercury hydroxide, oligo(dT)-primed double-stranded cDNA was synthesized (16) and then methylated with *Eco*RI and *Alu* methylases. The technique of Dorssers and Postmes (10) was used to introduce *Eco*RI and *Hin*dIII sites on the 5′ and 3′ ends of the cDNAs, respectively. After digestion with the *Eco*RI and *Hin*dIII restriction enzymes, cDNAs that were larger than 500 bp were isolated from an agarose gel by electroelution. The eukaryotic expression vector V19.10, predigested with *Eco*RI and *Hin*dIII, was ligated with the cDNA; ligation was followed by the transfection of competent *Escherichia coli* DH5α cells (GIBCO-BRL). The cDNA library was frozen in aliquots at −80°C after the addition of dimethyl sulfoxide to 7% (22).

A random-primed cDNA library was also constructed from the same mRNA templates. Briefly, the first-strand cDNA was synthesized with random hexamer nucleotides (LKB Pharmacia, Piscataway, N.J.) as the primers. An adaptor containing the partial *Bst*XI sequence (InVitrogen, San Diego, Calif.) was ligated to the double-stranded cDNA pool. Vector V19.12, which contains two nonpalindromic *Bst*XI sites, was digested with the *Bst*XI restriction enzyme and ligated to the cDNA. Transformation of host *E. coli* and the storage of the library were done as described above.

High-density screening of the oligo(dT)-primed peripheral blood lymphocyte library was carried out by plating about 10,000 colonies per 150-mm plate on a GeneScreen membrane (22). About 20 plates were screened with the oligonucleotide probe (see Results) for the signal peptidase cleavage site (see Fig. 1). After three rounds of screening, 80 clones were identified and sequenced, among which PBEF was one.

A commercial cDNA library of HUT 78 cells in lambda gt11 (Clontech Laboratories) was then screened by using the PBEF cDNA fragment from peripheral blood lymphocytes according to the manufacturer's protocol. The cDNA was sequenced after subcloning in bluescript SK II (Stratagene, San Diego, Calif.). A random-primed peripheral blood lymphocyte library was then screened to isolate an additional 5′ portion of PBEF cDNA. An oligonucleotide designed to represent the 5′-most portion of the original PBEF cDNA was used as the probe.

DNA sequencing and analysis. Plasmid DNA prepared by Qiagen technique was sequenced by the dideoxy chain termination method (27) by using synthetic primers. The DNA sequence was assembled and analyzed by the sequence analysis programs (25, 32) of the Genetics Computer Group (GCG) (University of Wisconsin, Madison) (version 7.2) on a Vax computer (Wordsearch, FASTA). BLAST and other GCG programs were used to search for homology of PBEF cDNA to sequences contained within the following data bases: GenBank (release 77.0), EMBL (release 35.0), and Swiss Protein (release 25.0). Structural analysis of PBEF protein was done by using the appropriate GCG programs. The Sigseq1 program was used to predict the signal peptide cleavage site (11).

Analysis of tissue-specific expression. A multiple-human-tissue blot containing RNA from brain, heart, kidney, liver, lung, pancreas, placenta, spleen, and testis tissues and skeletal muscle was probed with ^{32}P-labelled PBEF cDNA essentially according to the manufacturer's (Clontech Laboratories) protocol. Stripped blots were hybridized with labelled human β-actin probe to demonstrate that equal amounts of RNA were loaded in each well. Tissue-specific expression was also analyzed by using the reverse transcriptase (RT)-PCR technique. For RT-PCR, total RNA from human bone marrow was isolated with phenol-guanidine thiocyanate as previously described (6). Total RNA from heart, kidney, liver, spleen, and thymus tissues and skeletal muscle was bought from Clontech Laboratories. First-strand cDNA was synthesized from the above-mentioned RNA samples with RT (BRL Superscript). One-tenth of the first-strand cDNA was then used to amplify the PBEF gene by use of specific primers under stringent conditions. PCR products were run in a 0.8% agarose gel, transferred to a GeneScreen Plus (Du Pont, Wilmington, Del.) membrane, and hybridized to the PBEF internal probe to confirm the authenticity of the PCR products.

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5' GCC  TGC  AGC  ATC  TCT  GCA  CCC  GCC  CGC  TGC  CCC  GMCSF
5' CTT  GTC  ACA  AAC  AGT  GCA  CCT  ACT  TCA  AGT  TCT  IL-2
5' GCT  TAT  GTG  CAC  GAT  GCA  CCT  GTA  CGA  TCA  CTG  IL-1B
5' CCT  GCT  GCC  TCC  CCT  GCC  CCA  GTA  CCC  CCA  GGA  IL-6
5' CGC  CCC  GGA  CTC  CAA  GCT  CCC  ATG  ACC  CAG  ACA  IL-3

   5'at gtc  gac  MWC  SVT  GCM  CCH  RYM  YSM  YCY  A
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FIG. 1. Oligonucleotide probe design. An degenerate oligonucleotide was designed on the basis of the similarity in the coding sequences of GM-CSF, IL-2, IL-1β, IL-6, and IL-3 at the signal peptidase processing site. To represent degeneracy, the following abbreviations are used: M, either A or C; R, either A or G; W, either A or T; S, either C or T; Y, either C or T; V, either A, C, or G; H, either A, C, or T. A *Sal*I restriction site was added to the 5′ end of the oligomer for use in PCR.

Expression of PBEF as a fusion protein. For the production of antibodies, PBEF was expressed in *E. coli* as a fusion protein. The amino-acid-encoding portion of PBEF cDNA was ligated in frame to the 3′ end of a synthetic bovine growth hormone (BGH) coding sequence and was put under the control of the p_L promoter of plasmid pCFM 756 for expression. Competent *E. coli* FM5 cells were transformed with the fusion gene and were grown at 28°C until the optical density at 600 nm was 0.3 to 0.5. The temperature was then increased to 42°C for 2 to 3 h. Emergence of inclusion bodies was visualized with a microscope. *E. coli* cells were then lysed in Laemmli buffer. The cellular proteins were separated on a 10% polyacrylamide gel electrophoresis–sodium dodecyl sulfate (PAGE-SDS) gel and stained with Coomassie blue. A preparative PAGE was run, and the resulting fusion protein band was cut out, lyophilized, and injected into rabbits to raise polyclonal antibodies.

Transient expression of PBEF in COS 7 cells. About 3×10^6 COS 7 cells in phosphate-buffered saline (PBS) were transfected with V19.12 vector DNA containing PBEF cDNA by electroporation according to the manufacturer's (BTX, San Diego, Calif.) protocol. Cells were incubated overnight at 37°C in Dulbecco modified Eagle medium (GIBCO-BRL) containing 10% fetal calf serum and then were transferred to a serum-free medium. After 72 h, the conditioned medium was collected, filter sterilized, and frozen in aliquots at −20°C.

Stable expression of PBEF in PA317 cells. The cDNA for PBEF was inserted into the MPZen vector (4) for expression under a MPSV promoter. Psi 2 cells (19) were cotransfected with MPZen-PBEF DNA and SV2 Neo DNA. Colonies were selected on G418. Total RNA was isolated by the rapid phenol-thiocyanate method as described above and dot blotted to identify the best producer. This colony was grown to produce virus particles which were used to infect the amphotropic packaging cell line PA317 (19). Serum-free conditioned medium was generated from PA317 cells for protein purification and bioassays.

Purification of PBEF. PBEF was purified from the trans-

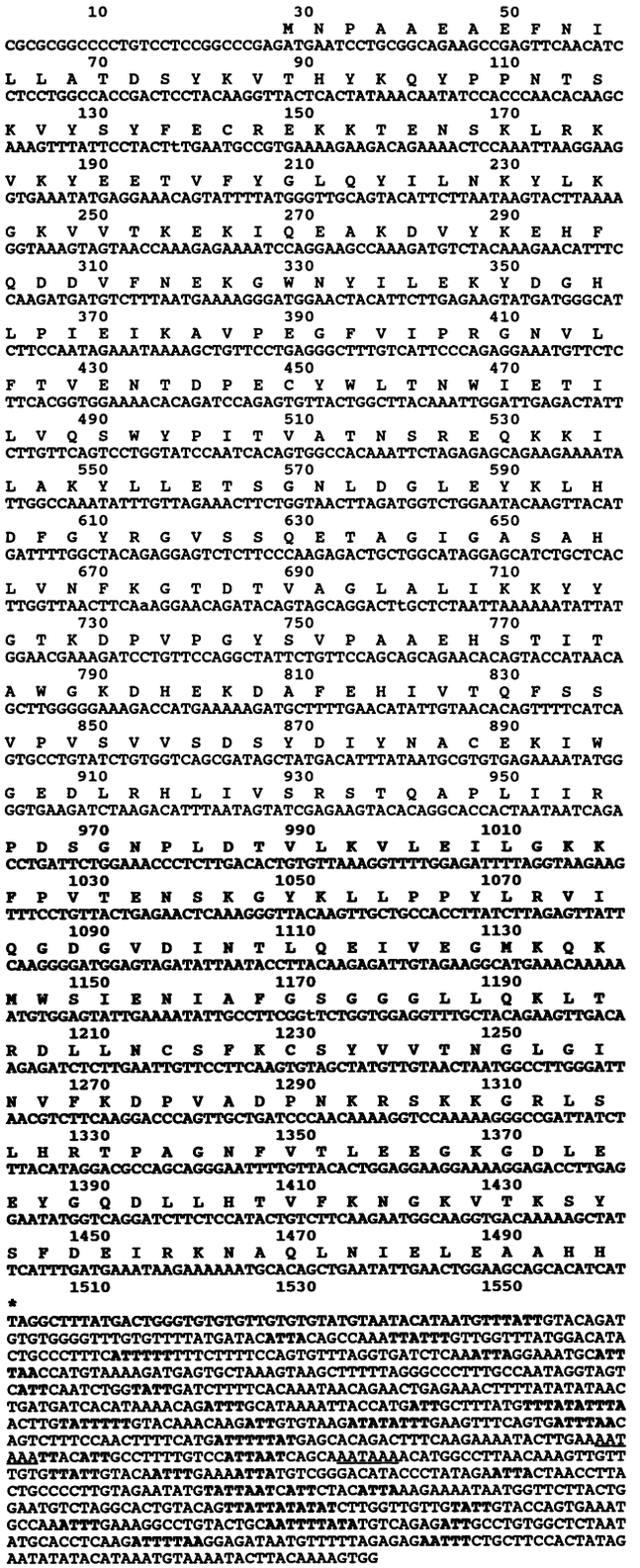

FIG. 2. Nucleotide and predicted amino acid sequences of PBEF. The nucleotide sequence of the coding strand is shown. Different clones for PBEF were sequenced on both strands with oligonucleotide primers. The consensus sequence of PBEF was obtained by aligning the sequences of different PBEF clones. Open reading frame assignment was done by using the MAP program of the GCG. TATT and other message-destabilizing sequences are in boldface type at the bottom. The polyadenylation signal, AATAAA, is underlined. *, end of the open reading frame.

fected PA317 conditioned medium by affinity chromatography on an antibody column. Polyclonal antibodies against the BGH-PBEF fusion protein were purified on an Affi-Gel protein A column (Bio-Rad, Richmond, Calif.) by following the manufacturer's protocol. The purified antibodies were coupled to cyanogen bromide-activated Sepharose essentially according to the protocol provided with the Immunopure antigen/antibody immobilization kit (Pierce, Rockford, Ill.). Conditioned medium from PA317 cells producing PBEF was applied to the antibody column, from which PBEF was eluted in ImmunoPure elution buffer, neutralized, and dialyzed against PBS before being used in different bioassays.

Colony forming assays. Bone marrow cells, obtained from normal adult BALB/c mice or mice treated with 5-fluorouracil, were plated in double-layer agar cultures in 35-mm dishes (3). The alpha modification of Eagle's minimal essential medium (Flow Laboratories) supplemented with 20% fetal calf serum was used for all cultures. Growth factors and/or conditioned media were incorporated in the underlay at a maximum of 13.2% of the total culture volume of 1.5 ml per dish. Cultures were gassed with a 5% O_2–10% CO_2–85% N_2 mixture and incubated for 10 to 14 days. Only colonies containing 50 or more cells were scored. Pre-B-cell colonies were identified as dense compact colonies and were verified to contain $B220^+$ cells by fluorescence-activated cell sorter (FACS) analysis.

RESULTS

PBEF is a novel cytokine-like molecule. A degenerate oligonucleotide probe was designed on the basis of similarity in nucleotide sequences surrounding and coding for the signal peptidase cleavage sites of a number of cytokines, i.e., granulocyte macrophage–colony-stimulating factor (GM-CSF), IL-1β, IL-2, IL-3, and IL-6 (Fig. 1). This probe could be used to isolate authentic cytokine genes with homology in the signal peptidase cleavage site. For example, we were successful in isolating IL-1β, IL-6, IL-8, and a number of chemokines. Clones with sequence homology or complements to this oligonucleotide probe in other parts of the genes were also isolated. In fact, PBEF belongs to the latter category.

In order to decide which novel clones to pursue, we analyzed the patterns of inducibility of these genes in peripheral blood lymphocytes in vitro. Most cytokine genes have been shown to be induced by lectins and superinduced by cycloheximide (33). In addition, the presence of destabilizing sequences such as TATT on the 3′ untranslated region of the clones, a salient feature of cytokine messages (28), was also taken into consideration. Clones, such as PBEF, which met these criteria were studied further. Isolation of full-length clones was followed by sequencing, expression, and analysis of the biological effects of their gene products in various bioassays. The PBEF clone was so designated after its product was found to be active in a pre-B-cell colony formation assay.

We were successful in isolating a number of clones of PBEF from the oligo(dT)-primed and random-primed periph-

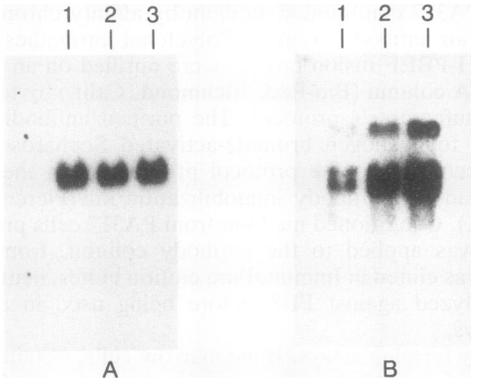

FIG. 3. Induction of PBEF in human peripheral blood lymphocytes by pokeweed mitogen. RNA was isolated from control lymphocytes as well as from lymphocytes which underwent pokeweed mitogen induction with or without cycloheximide. Northern blot analysis was done by using the end-labelled probe for either β-actin (A) or PBEF (B). RNA samples in lanes are as follows: 1, control lymphocytes; 2, pokeweed mitogen; 3, pokeweed mitogen and cycloheximide. Bands on the autoradiograph were quantitated with an Ultrascan XL densitometer (LKB-Pharmacia).

eral blood lymphocyte cDNA library as well as from a commercially obtained cDNA library which was constructed from phorbol myristate acetate-stimulated HUT 78 poly(A)$^+$ mRNA. The PBEF cDNA that includes both the coding and untranslated regions is 2.376 kb. There is only one open reading frame of 1,470 bases coding for a polypeptide of 52 kDa (Fig. 2). The 3′ untranslated region is 69% AT and contains multiple TATT motifs. There are two atypical polyadenylation signals, AATAAA, located 363 and 391 bases upstream from the 3′ end of our longest clone. In a smaller clone, the polyadenylation occurs only 47 nucleotides after the first polyadenylation signal. No significant homology for PBEF was found in existing data bases either at the nucleic acid or the amino acid level.

The mRNA for PBEF is represented in species of three different molecular sizes, i.e., about 2,000, 2,400, and 4,000 bp (Fig. 3 and 4). Either alternate splicing of exons or the use

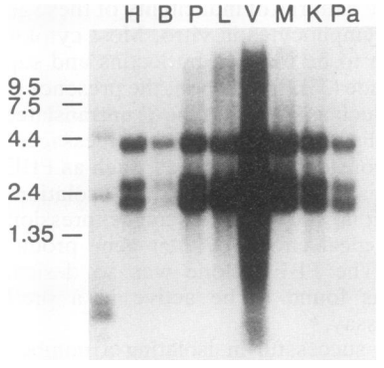

FIG. 4. Tissue specificity of PBEF mRNA expression. Multiple-human-tissue RNA blots bought from Clontech Laboratories were probed with random-primed PBEF. Equal amounts of RNA were loaded in each well and quantitated by hybridization with a human β-actin probe. Abbreviations for sources of RNA are as follows: H, heart; B, brain; P, placenta; L, lungs; V, liver; M, skeletal muscle; K, kidney; Pa, pancreas. Molecular size markers on the left are in kilobases.

of alternate polyadenylation sites could be the reason for this variation. In fact, we have obtained cDNA clones of both 2,000 and 2,376 bp which use two different polyadenylation sites about 400 bases apart. Rapid amplification of the cDNA ends and screening of the random-primed peripheral blood lymphocyte cDNA library did not provide clones with any significant additional 5′ end sequences (results not shown).

PBEF is induced by pokeweed mitogen and superinduced by cycloheximide. PBEF was further characterized for its inducibility by pokeweed mitogen. RNA was isolated either from control lymphocytes or from lymphocytes which were subjected to pokeweed mitogen activation with or without cycloheximide treatment. These RNA samples were hybridized on a Northern blot with random-primed PBEF cDNA. Hybridization to a β-actin probe was used to standardize RNA loading in the gel. Bands in autoradiograph were quantitated with an Ultrascan XL densitometer (LKB Pharmacia). As shown in Fig. 3, there was a four- to sixfold increase in the level of different-size messages after pokeweed mitogen treatment compared with that of the no-treatment control. The presence of cycloheximide during the induction process increased the message level an additional two- to threefold compared with that induced by pokeweed mitogen treatment.

PBEF is expressed in large amounts in bone marrow, liver tissue, and muscle. Tissue-specific expression of PBEF was determined both by Northern blotting and by RT-PCR. For Northern blot analysis, multiple-tissue RNA blots from Clontech Laboratories were hybridized with the random-primed PBEF cDNA clone (Fig. 4). The stripped blots were hybridized with a human β-actin probe to standardize loading of RNA into the gel (data not shown). The maximum amount of PBEF message was found in liver tissue, and the next highest amount was found in muscle tissue. In addition, it was also present in heart, placenta, lung, and kidney tissues. Similar results were also obtained from RT-PCR experiments in which PBEF was also found to be present in significant amounts in bone marrow (results not shown).

PBEF cDNA encodes a unique 52-kDa secreted protein. PBEF has only one large open reading frame, which encodes a polypeptide of 473 amino acids with an estimated molecular weight of 52,000. The PBEF preprotein lacks a typical signal sequence for secretion as determined from the Sigseq1 program, which is based on von Heijne's principle (31). However, the protein was found in the conditioned medium of activated lymphocytes as well as in HeLa cells by Western blot (immunoblot) analysis (results not shown). After transient expression in COS 7 cells and stable expression in PA317 cells, PBEF was also found in the conditioned medium, suggesting that it is a secreted protein (Fig. 5). We are now in the process of purifying enough protein from Chinese hamster ovary (CHO) cells to identify the amino terminus of mature PBEF.

The predicted protein has a hydrophobic amino terminus. There are six cysteine residues. The isoelectric point is 7.25. PBEF contains two sites for asparagine glycosylation, four potential protein kinase C phosphorylation sites, and five creatine kinase 2 phosphorylation sites.

To ensure that the deduced sequence indeed codes for a protein of 52 kDa, polyclonal antibody was raised against the PBEF protein. It was expressed in *E. coli* as a fusion with the amino terminus of BGH. The polyclonal antibody was able to detect a 52-kDa protein in the *E. coli* lysate after direct expression of PBEF (results not shown). In the conditioned medium of COS 7 and PA317 cells the antibody detected a band of around 52 kDa (Fig. 5). The lower-

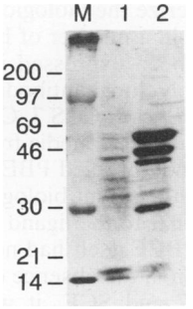

FIG. 5. Expression of PBEF in COS 7 cells. COS 7 cells were electroporated with V19.12 vector DNA containing PBEF cDNA. After 24 h, serum-free Dulbecco modified Eagle medium was added to the plates containing COS 7 cells. Conditioned medium was collected after 72 h of transfection and stored in aliquots after filter sterilization. Conditioned media from COS 7 cells transfected with either vector (lane 1) or vector containing PBEF (lane 2) were run on a 10% polyacrylamide–SDS gel (Novex, San Diego, Calif.) along with the molecular weight markers (in thousands) (lane M). Western blot analysis was carried out with the antibody raised against the PBEF-BGH fusion protein and developed by the enhanced chemiluminescence technique (Amersham, Arlington Heights, Ill.).

molecular-mass bands are possibly artifacts of the proteolytic degradation process.

PBEF enhances the effect of IL-7 and SCF on pre-B-cell colony formation. Conditioned medium from COS 7 cells transiently expressing PBEF was assayed in colony forming assays using normal bone marrow or mouse bone marrow that had been treated with fluorouracil. No colony formation was stimulated by PBEF when assayed alone. It did not have any stimulatory effect on primitive progenitor cells (HPP-CFC) from fluorouracil-treated bone marrow in the presence of either SCF, IL-3, granulocyte macrophage-CSF, or CSF-1. However, in the presence of both IL-7 and SCF, PBEF significantly enhanced the number and size of pre-B-cell colonies (Fig. 6). About 50 pre-B-cell colonies were formed in the presence of recombinant rat SCF (200 ng per culture) plus IL-7 (220 ng per culture) after 10 days of incubation. COS 7 cell conditioned medium containing PBEF increased the colony number by 70% without changing colony morphology. The conditioned medium from the vector control did not have any significant effect on the activity of SCF and IL-7. The number of colonies decreased at higher doses of the conditioned medium from transfected COS 7 cells. This inhibitory effect is common with crude conditioned medium and may not indicate the transmodulation of IL-7 and SCF receptors, as the affinity-purified PBEF did not have a similar inhibitory effect (see Fig. 8).

All colonies scored as pre-B-cell colonies had the typical dense compact appearance, and representative colonies showed the typical $B220^+$ phenotype after FACS analysis. The effect of PBEF on the increase in colony size was evident, but the percent increase could not be quantitated because of a high degree of heterogeneity.

Similar results were obtained with conditioned medium generated from PA317 cells carrying the PBEF gene in the retroviral vector MPZen (results not shown). To confirm that the pre-B-cell enhancing factor resides in the PBEF molecule, the factor was purified from the conditioned medium of PA317 cells by immune affinity chromatography. The isolated protein, when resolved on a 12.0% polyacrylamide gel, was represented by a single band after silver staining (Fig. 7). As shown in Fig. 8, there was a dose-dependent increase

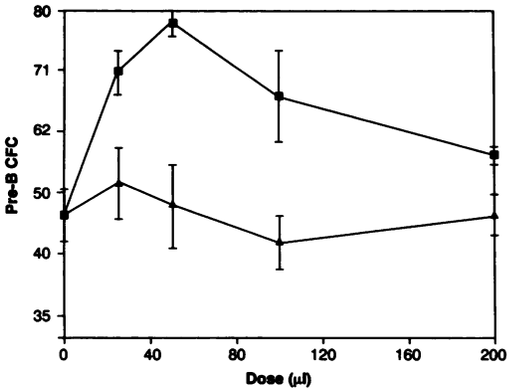

FIG. 6. Effect of PBEF on pre-B-cell colony formation. Conditioned media from COS 7 cells transfected with either the empty vector or the vector containing PBEF cDNA were tested for activity in a pre-B-cell colony formation assay with an agar culture of normal mouse bone marrow (see Materials and Methods for details). The amounts of conditioned medium, in microliters, are shown on the x axis, while the numbers of pre-B-cell colonies are shown on the y axis. Triangles indicate the numbers of pre-B cells in the presence of SCF–IL-7 and conditioned medium from the control, while squares indicate the numbers of pre-B-cell colonies in the presence of SCF–IL-7 and conditioned medium containing PBEF. The data are representative of data from 10 independent experiments.

in the number of pre-B-cell colonies in the presence of the purified PBEF without any decrease or plateau effect. There was up to a twofold increase in the number of colonies after the addition of purified PBEF to a final concentration of 2% (vol/vol).

We have not determined the effect of antibody on the colony formation, as the antibody was raised against the denatured PBEF-BGH fusion protein expressed in *E. coli* and most probably is not neutralizing.

DISCUSSION

Activation of peripheral blood lymphocytes by lectins such as phytohemagglutinin and pokeweed mitogen induces the expression of a number of known and unknown cytokine genes (33). We are in the process of isolating and characterizing the novel cytokine-like molecules by using multiple

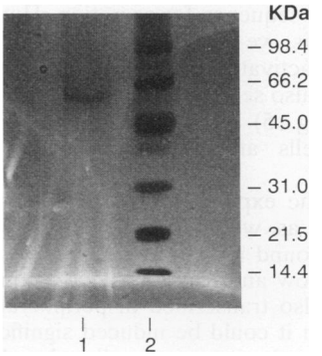

FIG. 7. Antibody-purified PBEF. PBEF was affinity purified from PA317 conditioned medium with polyclonal antibody, as described in Materials and Methods. The eluted material was dialyzed against PBS, analyzed on a 12.0% polyacrylamide–SDS gel, and silver stained. A single band of about 52 kDa is evident in lane 1. Lane 2 has molecular size markers (in thousands).

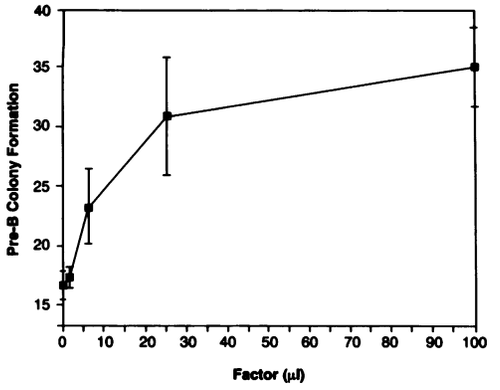

FIG. 8. Pre-B-cell colony enhancing assay. Pre-B-cell colony formation in the presence of IL-7–SCF and various amounts of purified PBEF in an agar culture of normal mouse bone marrow was determined as described in Materials and Methods. PBEF was purified from PA317 cells by affinity chromatography and dialyzed against PBS. Increasing amounts of PBEF was added to the mixture of SCF and IL-7 to quantify its effect on pre-B-cell enhancing activity. The data are a representative of those from four independent experiments.

approaches. One approach has been the use of a degenerate oligonucleotide probe, the sequence of which is shown in Fig. 1. A number of cytokines such as macrophage chemoattracting factor, PA374, IL-1β, IL-6, and IL-8 as well as a number of unknown genes from activated lymphocytes have been isolated by screening peripheral blood lymphocyte libraries with this probe. This report describes the characterization of one of these novel genes and its product.

This novel gene has been designated PBEF, as it enhances the effect of SCF and IL-7 on pre-B-cell colony formation. PBEF cDNA as well as the encoded protein is novel. We failed to find any homology with sequences present in either the GenBank, EMBL, or Swiss Protein data base at the nucleotide or amino acid level by using various programs, such as FASTA, TFASTA, or BLAST. The presence of multiple TATT and related message-destabilizing sequences on the 3′ untranslated region of the mRNA suggests that this gene could be coding for a cytokine-like molecule. Similar sequences have been shown to be present in labile messages of cytokines and oncogenes (28).

The PBEF mRNA encodes a novel polypeptide with a predicted molecular mass of 52 kDa. This polypeptide lacks a typical signal sequence for secretion. However, by Western blot analysis, we have shown that PBEF polypeptide is secreted from activated lymphocytes and from HeLa cells. The protein is also secreted from COS 7 cells after transient expression (Fig. 5) as well as from PA317, CHO, and baculovirus cells after stable transfection (results not shown).

Tissue-specific expression of PBEF was determined by Northern blot as well as RT-PCR analysis. The PBEF message was found mainly in human liver tissue, muscle, and bone marrow and to a lesser extent in lung and other tissues. It is also transcribed in peripheral blood lymphocytes, in which it could be induced significantly by lectins such as pokeweed mitogen as well as by phorbol myristate acetate. In this regard, the PBEF expression pattern resembles that of the polyfunctional molecule leukemia inhibitory factor, which is expressed in various tissues (18). Therefore, PBEF could be later found to have additional biological activities which have not yet been elucidated.

In order to characterize the biological activities of PBEF, we have expressed it in a number of heterologous systems. For example, it has been expressed in *E. coli* as a fusion protein to generate polyclonal antibodies. Direct expression has been done also in *E. coli*; COS 7, CHO, and PA317 cells; and baculovirus. Conditioned media from COS 7 and PA317 cells as well as antibody-purified PBEF from the latter cell line have been used in different biological assays.

We found that, similar to the ligand for the B-cell surface antigen CD 40 (15), PBEF itself had no detectable effect on any biological function in the absence of other cytokines. In the presence of IL-7 and SCF, it was very effective in increasing the pre-B-cell colony number and size. Therefore PBEF might play an active role in increasing the number of $B220^+$ cells for IL-7 to act on. The presence of an activity such as PBEF has been predicted before (9, 13), and in our assay conditions, the addition of insulin-like growth factor (IGF) did not substitute for the presence of PBEF.

No effect of PBEF on human pre-B cells has been found. This is probably due to the lack of effect of recombinant SCF and recombinant IL-7 on human pre-B-cell colony formation. We also failed to find any effect of PBEF on cells with myeloid or erythroid lineages.

Park and Osmond (23) also found that the levels of pre-B-cell proliferation and primary B-cell genesis normally taking place in mouse bone marrow may reflect the level of exposure to potential stimulants in the external environment. T cells help B-cell development by providing lymphokines which act as growth and differentiation factors for B cells (24). As PBEF is expressed after induction of the T-lymphoblastoid cell line HUT 78, it falls into this category of cytokines. At present we are further pursuing the characterization of the mechanism of PBEF action by using purified material from the baculovirus expression system.

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