Genomic organization of the gene coding for human pre-B-cell colony enhancing factor and expression in human fetal membranes

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ABSTRACT

Pre-B-cell colony enhancing factor (PBEF) was first isolated from an activated peripheral blood lymphocyte cDNA library and was found to be involved in the maturation of B-cell precursors. It was subsequently identified as one of the genes upregulated by distending the human fetal membranes *in vitro*. Here we report on the genomic organization of this gene, which is composed of 11 exons and 10 introns, spanning 34·7 kb of genomic DNA. Neither the gene nor the protein has any homology with other cytokines in any currently available database. The use of two promoters (proximal and distal) may result in differential, tissue specific expression of the PBEF transcripts. The 5′-flanking region lacks the classical sequence motif that would place it with the hematopoietic cytokines; however, it has several putative regulatory elements, suggesting that this gene may be chemically and mechanically responsive to inducers of transcription.

The three PBEF mRNA transcripts were observed in both normal and infected human fetal membranes but were significantly upregulated ($P<0.05$) in severe infection. The PBEF protein was immunolocalized, in both normal and infected tissues, to both the normal fetal cells of the amnion and chorion and the maternal decidua of the membranes, and to the invading neutrophils. These stained strongly and were likely to contribute to the increased expression in infection. The amniotic epithelial cell line (WISH cells) has been used as a model to study *PBEF* gene modulation. Lipopolysaccharide, interleukin (IL)-1β, tumour necrosis factor (TNF)α and IL-6 all significantly increased the expression of PBEF in 4 h of treatment. The addition of dexamethasone to IL-1β and TNFα significantly reduced the response of PBEF to these cytokines. IL-8 treatment failed to alter *PBEF* gene expression. Thus PBEF is a cytokine expressed in the normal fetal membranes and upregulated when they are infected. It is likely to have a central role in the mechanism of infection-induced preterm birth.

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INTRODUCTION

Pre-B-cell colony enhancing factor (PBEF) was first isolated from an activated peripheral blood lymphocyte cDNA library and was found to be involved in the maturation of B-cell precursors, in the presence of interleukin (IL)-7 and stem cell factor (SCF) (Samal *et al*.1994). Three PBEF mRNA transcripts of 2·0, 2·4 and 4·0 kb, with the 2·4 kb transcript predominating, were shown to be expressed in the bone marrow stromal cells, activated human lymphocytes, liver and muscle (Samal *et al*.1994). These trancripts were shown to be induced by pokeweed mitogen and superinduced by cycloheximide in peripheral blood lymphocytes. Using the T-lymphocyte cell line, HUT 78, PBEF mRNA expression was induced by phorbol ester treatment. The 52 kDa PBEF protein was expressed in the monkey kidney COS-7 and embryonic mouse fibroblast PA317 cells and was purified from the conditioned medium. This molecule had no activity alone on pre-B-cell colony formation, but it caused a 70% increase in the stimulation by IL-7 and SCF (Samal *et al*. 1994).

In an unrelated study to identify genes affected by the acute distension of the human fetal membranes, an amniotic epithelial cell line (WISH cells) grown on silastic sheets was distended for 4 h. Suppression-subtractive hybridization identified *PBEF* as a mechanically induced gene (Nemeth *et al*. 2000). When preterm and term full-thickness fetal membranes were similarly distended *in vitro*, it was shown by quantitative northern analysis that the *PBEF* gene was indeed upregulated. However, the degree of upregulation was greater in the preterm than in the term membranes, suggesting that by the end of gestation the membranes were less responsive, as they had attained their maximum distension and sensitivity to rupture *in vivo*, before their use *in vitro* (Nemeth *et al*. 2000). Thus it would appear that PBEF is involved in the normal physiological accommodation of the membranes to the rapidly growing uterus in late gestation (Millar *et al*. 2000).

There has been substantial progress in recent years in understanding the relationship between maternal infection and preterm birth (Goldenberg & Rouse 1998). The inflammatory response is induced by invading bacteria and mediated by polymorphonuclear neutrophils and macrophages, which invade the intrauterine tissues. These cells produce cytokines, matrix metalloproteinases and prostaglandins, all locally acting key components of fetal membrane rupture, cervical dilatation and uterine contraction (Parry & Strauss 1998). The distension of the fetal membranes, needed under normal circumstances, and the pathological response to local infection appear to be related via the production of certain cytokines. Their influence, in turn, on the production of the matrix metalloproteinases can be mediated by the normal resident cells of the tissue in the former instance and predominantly by the invading neutrophils and macrophages in the latter. Thus distension is likely to induce small controlled changes in the extracellular matrix remodeling enzymes, whereas infection would produce a more profound and harmful effect, resulting in membrane rupture.

A genomic clone (accession No. AC007032) recently deposited in the GenBank database by the Genome Sequencing Center, University of Washington School of Medicine, was found to match the cDNA sequence of *PBEF* (Samal *et al*. 1994). This gene was mapped on the long arm of chromosome 7 (7q22) near the D7S1552 marker within the 114·5 cM–120·7 cM interval. We report our analysis of the genomic sequence of *PBEF* and the promoter region, in order to gain insight into the molecular mechanisms of its regulation. Since the aberrant production of cytokines may lead to

disease states and cytokine production is strictly regulated (Taniguchi 1988), we show the increased expression of the *PBEF* gene in the fetal membranes with histologically identified chorioamnionitis and the PBEF protein localized in similarly infected tissues. The amniotic epithelial cell line (WISH cells) has been used as a model to study *PBEF* gene modulation by lipopolysaccharide (LPS) and the proinflammatory cytokines, IL-1β and tumour necrosis factor (TNF)α, in addition to IL-6 and IL-8.

MATERIALS AND METHODS

Computer analysis of the nucleotide sequence

Computer analysis to determine the exon/intron structure of the *PBEF* gene was performed using the Entrez and BLAST programs of the National Center for Biotechnology Information (NCBI, NIH, MD, USA) and BESTFIT and FINDPATTERNS programs of the GCG molecular biology software package (GCG-Wisconsin Package Version 9·1 Madison, WS, USA). The exon/intron junctions were predicted by comparing the cDNA from the genomic DNA sequence. Analysis of 5′-flanking and promoter regions of the *PBEF* gene was done using the Transcription Factor database (Ghosh 1996) and Promoter Prediction by Neural Network (http://www.fruitfly.org/seqtools/promoter.html).

Primer extension analysis of transcription initiation

Poly(A)$^+$ RNAs were prepared from the human amniotic epithelial cell line (WISH) (American Type Cultural Collection – CCL25, Manassas, VA, USA) grown in Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F12) supplemented with 10% fetal calf serum (Gibco Brl, Grand Island, NY, USA). The cells were grown to confluence at 37 °C in 95% O_2 and 5% CO_2. The poly(A)$^+$ RNAs were extracted using µMACS mRNA isolation kit (Miltenyi Biotec, Auburn, CA, USA). The two oligonucleotide primers (5′-GTCGGTGGCCAG GAGGATGTTG-3′ and 5′-GCCAGGAGGATG TTGAACTCGG-3′) were designed and selected using the GCG-Primer program (University of Wisconsin, USA) based on the *PBEF* genomic sequence. These primers were dissolved at a concentration of 100 µM and 5′-end labeled with [γ^{32}P]dATP (6000 mCi/ml; NEN Research Products, Boston, MA, USA) using T4 polynucleotide kinase (Gibco Brl). Labeled primers were purified using a Sephadex G-25 column. The labeled primers (5 pmol) were annealed to 5 µg

poly(A)$^+$ RNA using MuLV reverse transcriptase and reagents from PE Applied Biosystems (Foster City, CA, USA) and incubated at 23 °C for 10 min, 42 °C for 15 min and 94 °C for 5 min. The reverse transcribed cDNAs were analysed by electrophoresis using 8% polyacrylamide gel containing 6 M urea followed by autoradiography of the fixed/dried gel. The 50, 25 and 10 bp molecular size ladders were likewise labeled with [γ^{32}P]dATP using the T4 polynucleotide kinase labeling kit (Gibco Brl), then purified using a Sephadex G-50 column and used as size markers for the primer extension products.

Tissues

Human amnion, chorion and adhering decidua (fetal membranes), were collected at Kapiolani Medical Center for Women and Children (Honolulu, HI, USA) with informed consent and with approval from the University Committee on Human Experimentation and the Hospital Institutional Review Board. The fetal membranes were collected within 1 h of delivery and small rolls (1 × 1 cm) were placed in Bouin's fixative and embedded in paraplast (Fisher Scientific, Pittsburgh, PA, USA) for immunocytochemistry, or the entire membrane cut from the placental edge was placed into liquid nitrogen and kept at − 80 °C until used for the isolation of mRNA. For the immunolocalization of *PBEF*, three tissues from patients after normal term cesarean section before labor and three others from patients delivered at term with chorioamnionitis, diagnosed histologically by a pathologist, were collected and processed. Tissues for northern analysis were also evaluated by a pathologist and classified as normal ($n=6$), with inflammation of the maternal decidua (deciduitis) ($n=5$) defined as the mildest grade acute inflammation confined to the decidua, with moderate infection ($n=5$) defined as the infiltration of neutrophils showing some extension into the extracellular matrix, or with classical chorioamnionitis or severe infection ($n=3$) defined as the presence of abundant neutrophils within the fetal membranes (Salafia *et al*. 1989).

Immunolocalization

Tissue sections (7 µm) were cut and mounted on Vectabond-treated slides (Vector Laboratories, Burlingame, CA, USA), deparaffinized and hydrated in deionized water. They were treated with 0·3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity and washed in PBS for 20 min. Sections were treated

with normal goat serum (1·5%) for 20 min to block all non-specific binding sites and then incubated with a rabbit polyclonal antibody to human PBEF (Samal *et al*. 1994) at 5 µg/ml in 0·5% normal goat serum at room temperature for 30 min. The negative controls were adjacent sections processed with normal rabbit serum at the same concentration as the primary antibody. The sections were rinsed three times in 0·015 M PBS, 6 min total, then incubated with a biotinylated secondary antibody for 30 min, rinsed three times in 0·015 M PBS, 6 min total and treated with the ABC reagent (Vector Laboratories) for 30 min and diaminobenzidine (0·5 mg/ml) at room temperature for 7 min. The sections were washed in distilled water and counterstained with hematoxylin, dehydrated and mounted in Pro-Texx (Baxter Scientific, Honolulu, HI, USA) and viewed under bright field microscopy.

Human amnion-derived WISH cells (ATCC CCL25) were obtained from American type Culture Collection (Manassas, VA, USA). For immunostaining, 150 000 cells were grown in DMEM:F12+ 10% FBS on poly-L-lysine coated Nunc Chamber slides. After the cells reached 80% confluence, the medium was removed and the cells fixed in 95% ethanol for 5 min and stored at 4 °C until immunostained.

Northern analysis

Total RNA was extracted (Chomzynski & Sacchi 1987) and mRNA prepared (Aviv & Leder 1972). Samples of mRNA (10–20 µg) were electrophoresed, and the RNA transferred to a nylon membrane (Magna Graph, MSI, Westborough, MA, USA) as previously described (Millar *et al*. 1998). The cDNA probes were labeled using the Random Primed DNA labeling kit and following the manufacturer's instructions (Gibco Brl). The filters were hybridized at 68 °C for 1 h using Express Hyb solution (Clontech, Palo Alto, CA, USA). A final stringency wash in 0·1 × SSC, 0·1% SDS was performed at 50 °C. Autoradiography was carried out at − 70 °C using Kodak Max X-ray film and two intensifying screens. Filters were deprobed in 0·5% SDS for 10 min at 100 °C and hybridized sequentially with cDNA probes to PBEF, IL-6 and IL-8 and finally with a human glyceraldehyde-3 phosphate-dehydrogenase (G3 PDH) cDNA probe, as an internal standard. The northern blot was quantitated with an Ambis Image Acquisition and Analysis system (Ambis Inc., San Diego, CA, USA), using the G3 PDH value for each sample to standardize sample loading. Results were expressed as a ratio to G3 PDH. Statistical analysis was

performed using the Tukey–Kramer multiple comparison test.

For cells, total RNA was isolated by addition of Trizol (Gibco, Brl), following the manufacturer's instructions. Total RNA (15 µg) aliquots were denatured in glyoxal–DMSO and hybridized as for the mRNA. The hybridization signals were quantitated with an AMBIS densitometric system (Scanalytics, Billerica, MA, USA). After hybridization, the filter was deprobed by boiling in 0·5% SDS for 10 min. Confirmation of equal loading was based on the 28S rRNA signal and the results were expressed as a percentage of the control value. Analysis was performed using log transformed data and ANOVA followed by the Tukey–Kramer multiple comparisons *post-hoc* test (GraphPad Software, Inc.).

The cDNA probe for *PBEF* (600 bp) was prepared as described previously (Nemeth *et al.* 2000). The IL-6 cDNA probe (639 bp) was prepared in our laboratory as previously described (Millar *et al.* 1998) and the IL-8 cDNA probe (425 bp) was a generous gift from Dr Kouji Matsushima, Cancer Center, Kanazawa, Japan.

Amniotic epithelial (WISH) cell culture and treatment

The cells were cultured in Dulbecco's Modified Eagle's Medium:Ham F-12 (DMEM:F12) (1:1) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA), penicilin (50U/ml)–streptomycin (50 µg/ml) and incubated at 37 °C in 5% CO_2/95% air. After reaching confluence, the cells were trypsinized and plated into six-well culture dishes at a density of $1 \times 100\,000$ cells in 4 ml medium. After 48 h or when the cells reached 80% confluence, the growth medium was replaced with minimal medium (0·5% FBS and 0·2% lactalbumin in DMEM:F12) and incubated for 12 h. Treatment of the cells began with addition of agents prepared in minimal medium. Recombinant human TNFα, IL-1β and IL-8 were obtained from R&D Systems, Inc. (Minneapolis, MN, USA) and LPS, *E. coli* serotype 055:B5 and dexamethasone 1 µM (water soluble) were obtained from Sigma Chemical Co. (St Louis, MO, USA). All treatments were carried out in triplicate and each experiment was performed at least three times on different occasions. Controls received minimal media containing 0·5% BSA (15 µl) in PBS. After incubation with treatments, the media were aspirated and the cells lysed in 1 ml Trizol reagent (Life Technologies, Grand Island, NY, USA). Total RNA was isolated following the manufacturer's procedure.

RESULTS

Gene organization and analysis of *PBEF* 5′-flanking region

The *PBEF* gene was found within a 126·8 kb genomic clone (BAC clone RP11–22N19) sequenced at the Genome Sequencing Center, University of Washington School of Medicine and submitted to GenBank by Dr R H Waterston. The *PBEF* gene is estimated to span a length of 34·7 kb and is composed of 11 exons and 10 introns. The general exon/intron organization of this gene is shown in Fig. 1A and was obtained by comparing the cDNA and genomic DNA sequences using the BLAST program and confirmed by using the BESTFIT and FINDPATTERNS programs (GCG-Wisconsin Package). Exon 1 encodes a short 5′-untranslated region (UTR) and the signal peptide region, whereas exon 11 encodes the carboxyl end of the PBEF protein and all of the 3′ UTR. The GT and AG consensus splice junction sequences were well conserved at all of the exon/intron boundaries. No other open reading frames were found, other than that previously published (Samal *et al.* 1994).

Through primer extension analysis using mRNA from the amniotic WISH cell line, multiple transcription initiation sites were observed. The major transcription initiation site designated as +1 (Fig. 1B), was located 50 bp upstream from the translation initiation codon (ATG) and 22 bp upstream from the reported cDNA sequence (Samal *et al.* 1994). There were several minor transcription initiation sites within 200 bp upstream of the major transcription initiation site (data not shown).

Sequences at the 3·2 kb 5′-flanking region upstream of the transcription initiation site and the introns were compared with known regulatory elements using the Transcription Factor Database (Ghosh 1996). Several types of putative regulatory elements were identified and are shown in Fig. 1B. The 5′-flanking region can be distinctly divided into two segments: the proximal 1·4 kb is more GC rich (60% GC), whereas the distal 1·6 kb has more AT bases (60% AT). Within the proximal region there is an extremely G+C-rich segment (72% GC) spanning the first 500 bp upstream of the major transcription initiation site, which lacks canonical TATA and CAAT boxes and contains several transcription initiation sites. In the distal region, however, several CAAT boxes and TATA-like and initiator sequences were found about 2 kb upstream from the transcription initiation site. This distal segment may act as a distal promoter (Fig. 1B). Multiple Sp1 binding sites (GC-boxes) were found all along the 5′-flanking region; 12 of these were located at the predicted proximal promoter region.
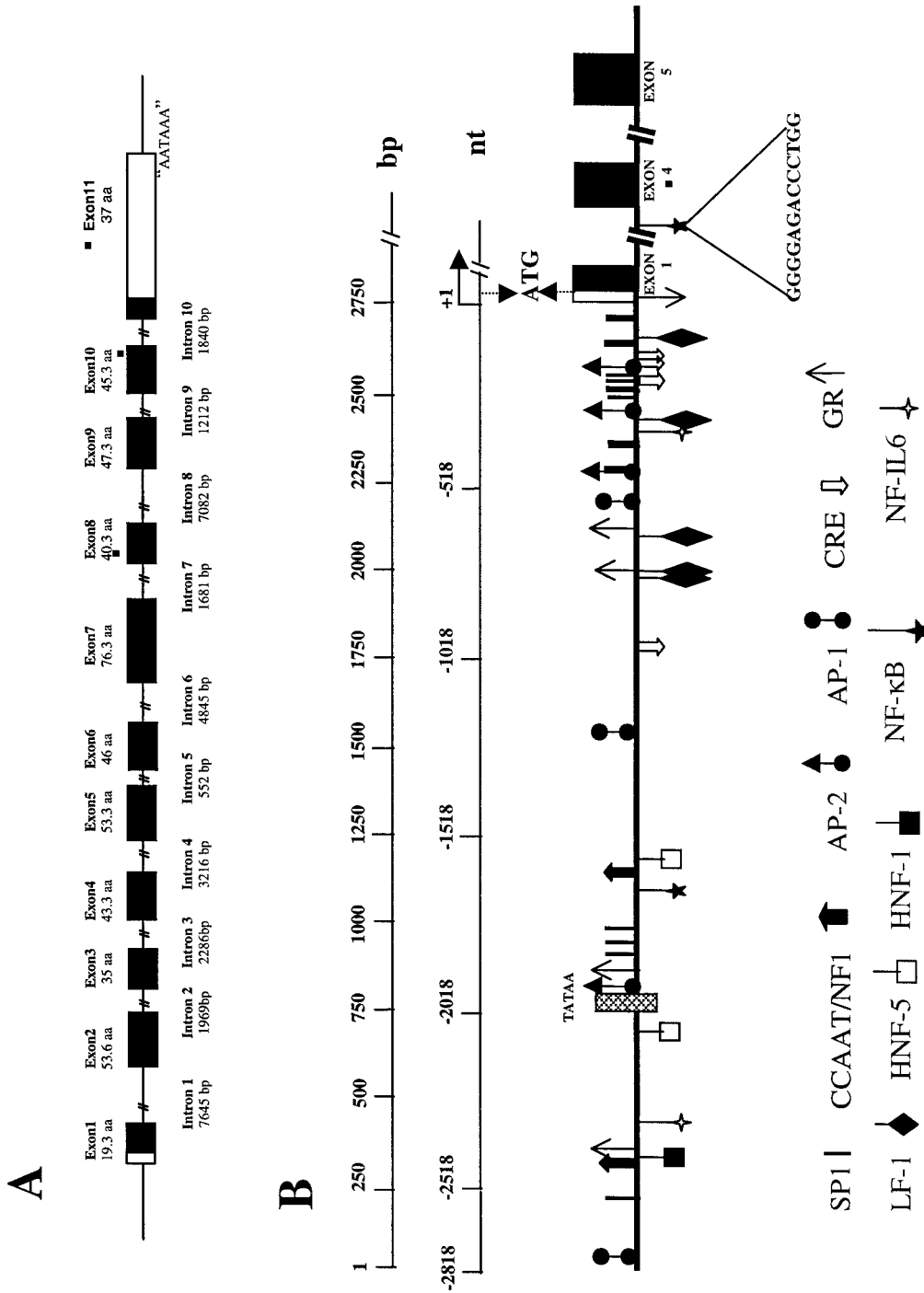
FIGURE 1. Genomic structure of the human *PBEF* gene and identification of regulatory elements. (A) Exon/intron organization of the human *PBEF* gene. The exons are shown as rectangles with the coding and non-coding regions indicated by solid and open boxes, respectively. The 11 introns are shown as lines with two vertical bars to indicate that the entire lengths are not included. The length of exons is given as the number of amino acids for which they are coding and the length of the introns is in base pairs. (B) Schematic representation of regulatory elements in the 5'-upstream region. The line marked bp (base pairs) shows the length of the analyzed 5'-region; that marked nt (nucleotides) shows the distance from the transcription initiation site. Exons 1, 4 and 5 are shown as solid boxes. The transcription initiation site is marked as +1 and was used as the reference nucleotide to position the regulatory elements. Symbols used to represent these *cis* elements are shown below the diagram. The hatched box represents one of the TATA boxes; this was followed by the initiator sequence (not shown). An NF-κB binding element in the third intron, which possibly confers the stretch responsiveness, is shown with the conserved motif.
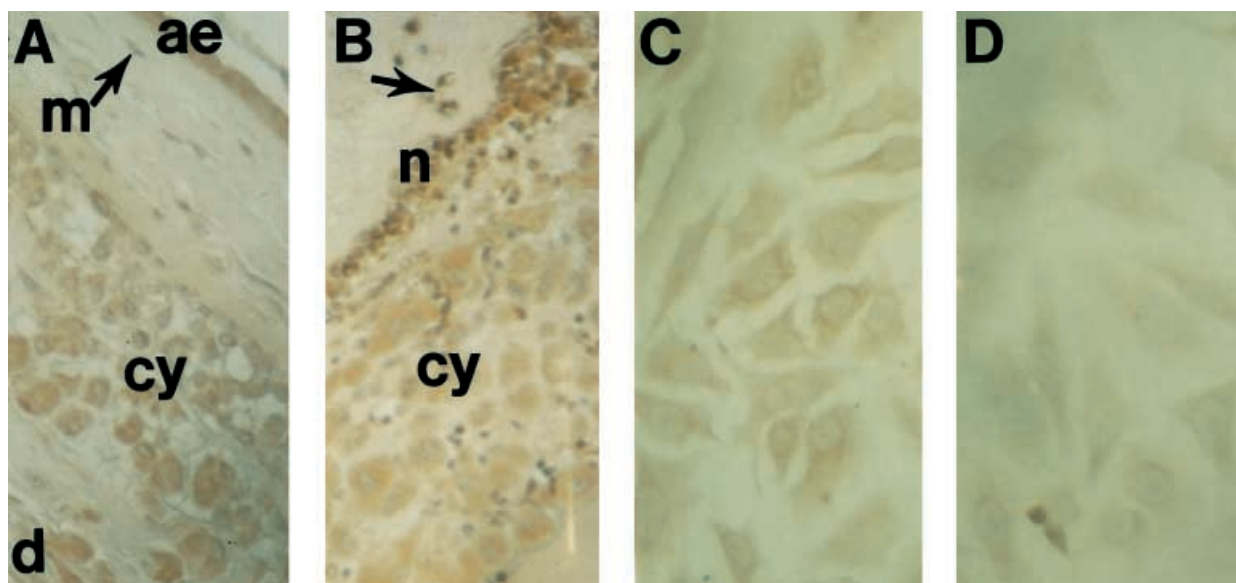
FIGURE 2.   Immunolocalization of PBEF in the human fetal membranes and in WISH cells. Staining was with a rabbit antiserum to PBEF. (A) Fetal membrane from a normal patient delivered at term by cesarean section before labor. This shows staining in the cells of the amniotic epithelium and the mesenchyme of the connective tissue, in addition to the cells of the chorionic cytotrophoblast and the decidua. (B) Fetal membrane from a patient with chorioamnionitis showing the cells of the amnion and chorion stained as well as a dense infiltrate of neutrophils beginning to invade the chorionic connective tissue (arrow); these are stained heavily for PBEF, in contrast to the cells of the cytotrophoblast. ae, Amniotic epithelium; cy, chorionic cytotrophoblast; d, decidua; m, mesenchyme; n, neutrophils. (C) The cytoplasm of WISH cells stained for PBEF; compare with the control (D) (original magnifications, × 300).

In addition to the Sp1 sites, binding sites for the ubiquitous transcription factors such as the CCAAT/nuclear factor 1 (NF-1), AP-1 and AP-2, were also identified. The binding sites for NF-1 were concentrated in the putative distal promoter region, those for AP-2 were mainly in the proximal promoter region, and AP-1 sites were uniformly distributed. In addition, the hormonally and chemically responsive regulatory elements, which include the binding sites for the glucocorticoid receptor, corticotropin releasing factor, cAMP response element binding protein (CREB) and the nuclear factors such as NF-IL6, were present in both proximal and distal promoter regions. NF-κB, another hormonally responsive element, was present only in the distal promoter region. Analysis of the introns showed the presence of the NF-κB binding site (GGGAGGCCC) localized in the third intron, whereas introns 5, 8, 9 and 10 had NF-κB-like elements (GGGAGGCXX) present. Another group of regulatory elements that bind tissue specific transcription factors, such as liver factor-1 and hepatic nuclear factors were also identified (Fig. 1B).

The sequence motif 'GPuGPuTTPyCAPy' – which is well conserved in the 5′-flanking regions of hematopoetic cytokines, including IL-2, IL-3, granulocyte-macrophage colony-stimulating factor (Stanley *et al*.1985) and granulocyte-colony stimulating factor (Nagata *et al*. 1986) and is considered a diagnostic feature – was not present in the *PBEF* gene.

Immunolocalization

PBEF was immunolocalized to the fetal cells in the amnion and chorion and in the adhering maternal decidual cells. An example of a normal term tissue is shown in Fig. 2A. The cells of the amniotic epithelium and the mesenchymal cells embedded in the extracellular matrix of the amnion were stained. The rounded cells of the chorionic cytotrophoblast and the more elongated cells of the decidua also were stained. However, chorioamnionitis or infection of the fetal membranes is always associated with the infiltration of neutrophils from the maternal decidual side of the membrane (Blanc 1959). Thus the infected tissue shown in Fig. 2B has a dense infiltrate of neutrophils beginning to invade the chorionic connective tissue. These neutrophils stained heavily for PBEF and are shown in contrast to the lighter staining of the cells of the chorionic

cytotrophoblast. We originally cloned the *PBEF* gene from WISH cells; however, the protein is shown immunolocalized here to the cytoplasm of these cells (Fig. 2C); the respective control with very light staining is shown in Fig. 2D.

Expression of mRNA

All three transcripts of *PBEF* were present in the human fetal membranes as shown in the northern blot in Fig. 3A. There was no increased expression of *PBEF* in the tissues exhibiting inflammation or moderate infection; however, *PBEF* expression was significantly increased in the tissues from the patients with severe infection compared with those from the normal patients ($P<0.05$) (Fig. 3B). All three *PBEF* transcripts were equally upregulated in the infected tissues. There was considerable patient-to-patient variation in the amount of *PBEF* expressed, and this was also the case for the expression of IL-6 and IL-8 on the same northern blot (Fig. 3C, D). The expression of IL-8 was significantly greater ($P<0.05$) in the severely infected tissues than in the normal, inflamed or moderately infected tissues. Although IL-6 expression was increased in the severely infected tissues, this did not reach statistical significance. The overall expression of PBEF in these samples correlated well with that of both IL-6 ($r=0.88$) and IL-8 ($r=0.99$), showing that the level of cytokine activation in the different tissues was under similar control.

Effects of LPS and cytokines on the expression of *PBEF* by amniotic epithelial WISH cells

The amniotic epithelial cell line, WISH, was used to study the modulation of *PBEF* expression by LPS treatment and by treatment with a selection of cytokines. The cells were treated in triplicate with two doses of LPS (50 and 500 ng/ml) for 2, 4 and 24 h. The expression of *PBEF* was assessed by northern blotting of the total RNA isolated from the cells at the termination of treatment. This was calculated as the degree of upregulation (fold) compared with the respective control at each time (Fig. 4). The expression of PBEF increased significantly at 4 h after treatment with both doses of LPS ($P<0.001$). There was a further significant ($P<0.01$) increase in *PBEF* gene expression with 500 ng/ml LPS at 24 h treatment (Fig. 4).

WISH cells were then treated with two pro-inflammatory cytokines, IL-1β at three concentrations (0.1, 1.0 and 10 ng/ml) for 4 h, with and without the addition of 1 µM dexamethasone
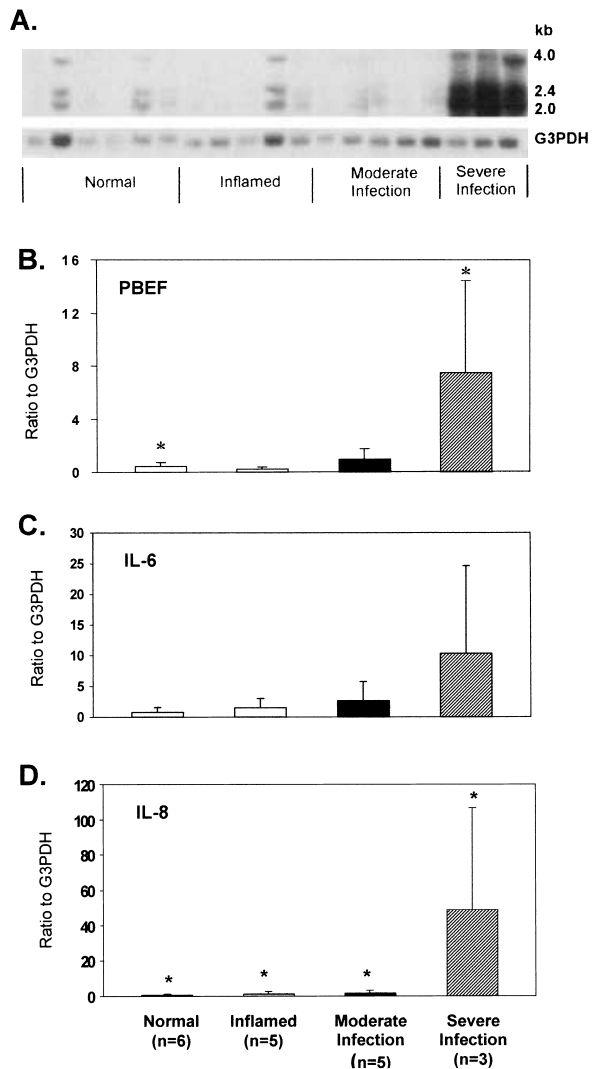

FIGURE 3.   Expression of *PBEF*, IL-6 and IL-8 mRNA in the human fetal membranes. (A) Northern blot of *PBEF* with mRNA from fetal membranes from normal term patients ($n=6$) and those with inflammation of the decidua ($n=5$), moderate infection ($n=5$) and severe infection ($n=3$). Three transcripts of *PBEF* were present in all tissues. Directly beneath is the G3 PDH on the same filter. (B) Quantitation of the northern blot for expression of *PBEF*. (C) Quantitation of IL-6. (D) Quantitation of IL-8. *Significantly ($P<0.05$) increased expression of *PBEF* in the severely infected tissues compared with the normal samples. The expression of IL-8 was significantly ($P<0.05$) increased in the severely infected tissues compared with the normal, inflamed or moderately infected tissues. The levels of IL-6 expressed in the severely infected tissues did not reach significance compared with the other groups.
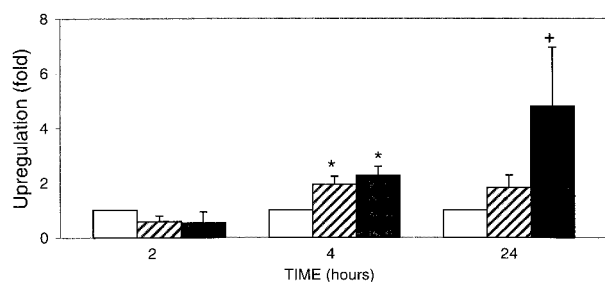
FIGURE 4.   Effect of LPS 50 ng/ml (hatched bars),
500 ng/ml (solid bars), and controls (open bars), on
PBEF gene expression in WISH cells. Significant effect
(*$P<0.001$) at 4 h with both doses of LPS, and with a
further significant increase (+$P<0.01$) in expression with
500 ng/ml by 24 h of treatment.

significantly increased expression of *PBEF*
($P<0.001$) compared with the control. The addition
of dexamethasone to all doses of IL-1β significantly
reduced the responses ($P<0.01$), and its addition to
TNFα-treated cells caused a significant ($P<0.05$)
reduction in *PBEF* expression at 0.05 ng/ml TNFα
and a significant ($P<0.01$) reduction at the two
greater doses of TNFα (Fig. 5A and B). Because
PBEF gene expression was increased by the
addition of these proinflammatory cytokines, we
then sought changes in its expression with the
addition of IL-6 and IL-8 (0.1, 1.0 or 10 ng/ml)
for 4 h. There was a significant increase ($P<0.001$)
in the expression of *PBEF* in response to the
addition of 1.0 and 10 ng/ml IL-6 (Fig. 6A). The
higher dose of IL-6 caused a significantly greater
($P<0.01$) effect than the lower dose of 1.0 ng/ml
(Fig. 6A). There was, however, no effect of any of
the doses of IL-8 on the expression of *PBEF*
when compared with the control (Fig. 6B). This
was an unexpected result and was, therefore,
repeated several additional times on separate
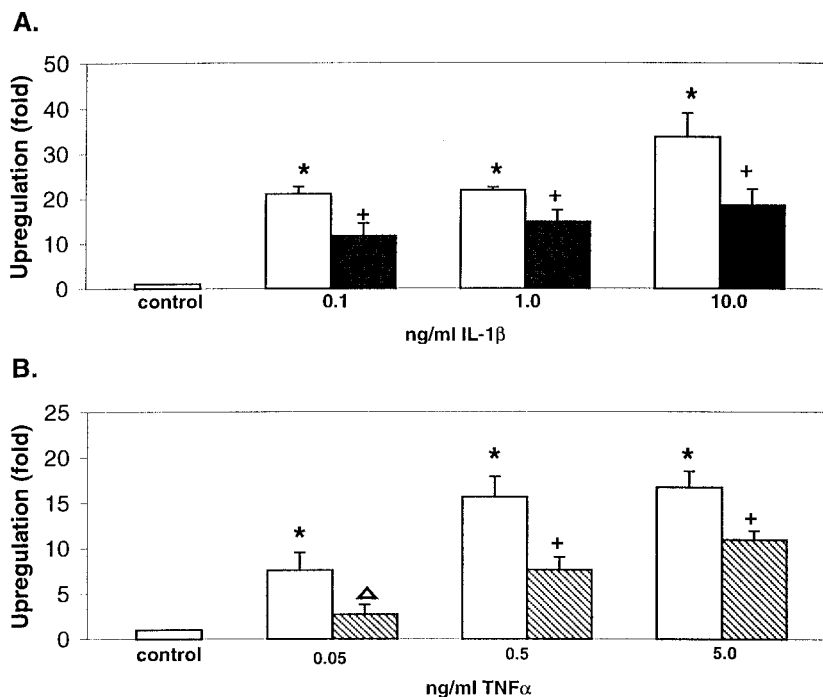occasions using fresh preparations of IL-8, with
identical results.

(Fig. 5A), or TNFα at 0.05, 0.5 and 5.0 ng/ml for
4 h, with or without the addition of dexamethazone
(Fig. 5B). The minimum doses of IL-1β and TNFα
caused significant increases in the expression of
PBEF ($P<0.001$) in the 4-h treatment. Each of the
higher doses of IL-1β and TNFα likewise caused

FIGURE 5.   Effects of IL-1β (A, solid bars) and TNFα (B, hatched bars) with
1 μM dexamethasone on *PBEF* gene expression over 4 h in WISH cells. Open
bars, without 1 μM dexamethasone. IL-1β and TNFα significantly (*$P<0.001$)
increased *PBEF* gene expression at all doses compared with the control.
Dexamethasone in all doses significantly reduced the effects of IL-1β
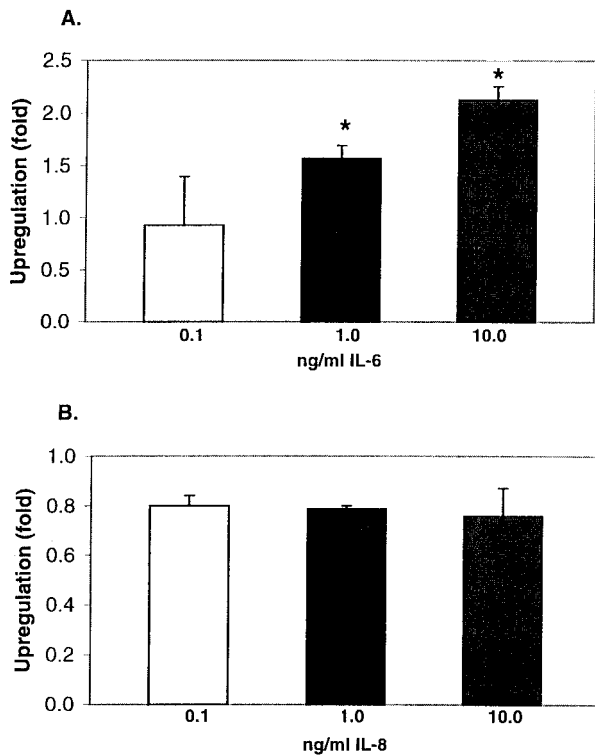(+$P<0.01$) and TNFα (△$P<0.05$; +$P<0.01$).

FIGURE 6.   Effects of IL-6 and IL-8 on *PBEF* expression in WISH cells. (A) IL-6 treatment of WISH cells for 4 h caused a significant (*$P<0.001$) increase in *PBEF* gene expression compared with the control at 1·0 and 10 ng/ml. (B) IL-8 treatment of WISH cells for 4 h had no effect on *PBEF* gene expression at any dose.

DISCUSSION

In this paper we have described the structure of a novel cytokine gene, termed pre-B cell colony-enhancing factor (*PBEF*) and shown it to be expressed in the human fetal membranes in normal pregnancy and significantly increased in the presence of infection.

There are three PBEF mRNA transcripts: 2·0, 2·4 and 4·0 kb. Through primer extension experiments we have identified multiple transcription intiation sites within the proximal 200 bp region. Thus the use of alternative polyadenylation signals in the 3′-UTR (Samal *et al*. 1994) in combination with alternative use of the major, or one of the minor transcription initiation sites probably contributes to the 2·0 and 2·4 kb transcripts. The conditions used for this analysis, however, did not allow us to determine the presence of longer transcripts corresponding to the 4·0 kb mRNA species. Samal *et al*. (1994) previously suggested that an alternative splicing of an exon/intron would

give rise to the 4·0 kb PBEF mRNA. Sequence analysis of the *PBEF* gene, however, showed that all the exon/intron splice junctions conformed to the AG/GT rule. Moreover, no other open reading frame was found, thus it is unlikely that alternative splicing of this gene occurs.

Computer analysis of the 5′ upstream sequence showed the presence of TATA and CAAT boxes, in addition to initiator sequences, at 2 kb upstream from the major transcription initiation site, suggesting the use of a distal promoter for the expression of the 4·0 kb PBEF mRNA. The presence of multiple promoters has been reported for many genes, including those for the parathyroid hormone (PTH)/PTH-related protein receptor, PTH-related protein (McCuaig *et al*. 1995, Joun *et al*. 1997) and human calcium-sensing receptor (Chikatsu *et al*. 2000). A structural organization very similar to that of the *PBEF* gene has been reported for the gene for human gonadotropin-releasing hormone receptor (*hGnRH-R*) (Ngan *et al*. 2000), which has a proximal promoter with high G+C content and no TATA or CAAT boxes and a distal promoter with multiple TATA and CAAT boxes.

Quantitative northern analysis showed all three *PBEF* transcripts were present in the human fetal membranes. It is possible that, in other tissues, the use of one or both promoters may result in the differential expression of the PBEF mRNAs. Moreover, the alternative use of these promoters may result in a tissue-specific expression of the *PBEF* gene, in a manner similar to that reported for the *hGnRH-R* gene (Ngan *et al*. 2000).

Regulatory elements such as NF-1, AP-1 and NF-κB, which are important for the transcriptional activation of cytokines, were found in the *PBEF* gene, with NF-κB located only in the distal promoter region. In contrast to other cytokines, AP-2 was represented at multiple sites, located predominantly in the proximal promoter. The presence of AP-2 and CREB sites suggests that the proximal promoter is more susceptible to the regulation by hormones and phosphorylation pathways. Glucocorticoids inhibit the production of cytokines at the pre-translational level (Lew *et al*. 1988). The *PBEF* gene contains several gluco-corticoid response elements (GREs), which are apparently functional, as dexamethasone was shown, here, to inhibit the IL-1β- and TNFα-induced transcription of *PBEF* in amniotic epithelial WISH cells. This suggests that dexamethasone may act directly on the *PBEF* gene through the GREs, in addition to the antagonism with NF-κB, as described for other cytokines.

Our primary interest in the analysis of the 5′-flanking region of the *PBEF* gene was to gain

insight into the mechanisms of its upregulation by both infection and mechanical distension of the fetal membranes. *PBEF* transcription was shown to be acutely upregulated by the addition of IL-1β, TNFα, and IL-6 to amniotic epithelial WISH cells; however, IL-8 had no such effect. This is consistent with the presence of IL-6 response elements in both the proximal and distal promoter regions of the *PBEF* gene, thus contributing to the effect of IL-6. In contrast, no IL-8 response elements were found and there was no effect of IL-8 on the transcription of *PBEF*. We have shown an increase in the transcription of *PBEF* in severely infected fetal membranes, suggesting that *PBEF* may be regulated similarly to IL-6 (Grassl *et al*. 1999) and IL-8 (Kunsch *et al*. 1994). When the 5′-flanking regions of these three genes were compared, binding sites for NF-κB, AP-1 and NF-IL6 were found, whereas a cAMP response element was present only in IL-6 and *PBEF*. The single NF-κB response element in the 5′-flanking region of the *PBEF* gene, and the variant found in the third intron and NF-κB-like elements present in introns 5, 8, 9 and 10, may be important for the regulation of this gene by mechanical distension (Nemeth *et al*. 2000), as NF-κB elements appear to be responsive to mechanical stimulation (Chaquour *et al*. 1999). The latter authors showed that the expression of a transfected reporter gene bearing a 1·1 kb fragment of the promoter of the platelet-activating factor receptor gene (*PAF-R*) was enhanced in mechanically stretched pulmonary artery smooth muscle cells, indicating a direct effect on transcriptional activity. When this was truncated, the promoter–reporter construct lost this stretch inducibility (Chaquour *et al*. 1999). This region in the *PAF-R* gene contained four copies of NF-κB binding sites in close proximity to each other. In the *PBEF* gene, there was a single NF-κB site in the 5′-flanking region and another in the third intron, the sequence of which exactly matched the sequence of one in the *PAF-R* gene (Chaquour *et al*. 1999). Additional NF-κB-like elements in the introns may also contribute to the induction of *PBEF* expression through distension.

During infection of the fetal membranes, there is invasion of the decidua, chorion and amnion by bacteria, accompanied by the release of bacterial LPS and other bacterial products, and this is associated with a marked infiltration by neutrophils, macrophages and other leukocytes as part of the host response to the infection (Quinn *et al*. 1987). We found that, in the presence of infection, the invading neutrophils stained darkly for PBEF and probably contribute to the increased transcription of *PBEF* in infected tissues. A fourfold change in *PBEF* expression was noted when human fibroblasts were exposed to cytomegalovirus, resulting in a direct alteration of *PBEF* gene expression (Zhu *et al*. 1998). Therefore, in infected fetal membranes, both the resident tissue cells and the invading inflammatory cells are likely to be sources of increased *PBEF* expression.

The action of IL-8 as a chemokine has been shown to be critical to the process of parturition in the human (Osmers *et al*. 1995) and its association with preterm delivery has also been reported (Wennerholm *et al*. 1998). Our finding that both PBEF and IL-8 increased on the stretching of the human fetal membranes (Nemeth *et al*. 2000) suggests a possible relationship between these cytokines in the process of human birth. From these analyses, it is likely that PBEF is indeed a cytokine, which lacks homology, at either the nucleotide or amino-acid level, with the other human cytokines in the current databases. Although the sequence motif diagnostic for most hematopoetic cytokines was absent, regulatory elements important for the transcriptional activation of cytokines in general have been identified. The abundance of the regulatory elements within its 5′-flanking region reflects a gene that is strictly regulated – common among cytokines (Taniguchi 1998). It is likely that *PBEF* will be found to have a central role in the mechanism of infection-induced preterm birth, which is a most serious problem in obstetrics today (Goldenberg & Rouse 1998).

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