Platelet basic protein (PBP) is a chemokine family member that is only found in platelets and their precursors megakaryocytes. The PBP gene is physically linked to the gene for another platelet-specific chemokine, platelet factor 4. While the biological basis of platelet factor 4 expression has been pursued by others, the regulatory features controlling the platelet-specific expression of PBP have not been investigated. In this article, we examined the molecular basis by which this megakaryocyte-specific gene is regulated. Transient expression studies of truncated reporter constructs containing from 4.5 to 0.1 kilobases of the functional PBP gene 5'-flanking region, demonstrated that the proximal 0.1 kilobases of the promoter was sufficient for high levels of expression in human erythroleukemia and CHRF-288 cells, two megakaryocytic cell lines. However, none of these constructs was expressed above background levels in HeLa and 293 cells, two non-megakaryocytic cell lines. Further truncation of this promoter suggested that there was an important regulatory element(s) within a pyrimidine-rich tract. Mobility shift analysis of the pyrimidine-rich tract defined a region between −85 and −64 which bound to a nuclear factor(s). This region contains sequences matching the consensus Ets-binding site from −78 to −75 base pairs. In particular, we noted that this site matched a PU.1 consensus sequence known as a PU box. Mobility shift and supershift studies with nuclear extracts as well as recombinant PU.1 protein and anti-PU.1 antibody further confirmed that PU.1 was the specific Ets family factor that bound to this site. Transient expression assays using reporter constructs which contained point mutations that abrogated PU.1 binding also significantly reduced PBP promoter activity in human erythroleukemia and CHRF cells. In addition, while all reporter gene constructs containing PBP promoters were completely inactive in HeLa cells, transactivation experiments using a PU.1 expression construct demonstrated that exogenous expression of PU.1 could increase reporter gene expression up to 8-fold in these cells. Finally, the role of PU.1 in PBP gene expression was compared between wild-type and PU.1-null embryonic stem (ES) cells that were differentiated in vitro into cells that resembled megakaryocytes both morphologically and immunologically. We found that PBP gene expression in the differentiated PU.1−/− null ES cells (as determined by semi-quantitative reverse transcriptase-polymerase chain reaction) was more than four times lower than that in the wild-type ES cells, while other platelet-specific genes were expressed equally or similarly in the two ES cell lines. Previous reports have shown that PU.1 is expressed in several hematopoietic lineages, including megakaryocytes. However, the functional role of PU.1 has only been previously demonstrated in the myeloid and lymphoid lineages. Therefore, our studies are the first to show the biological importance of this nuclear factor in the regulated expression of a megakaryocyte-specific gene.

Circulating platelets play a major role in thrombus formation at the site of tissue injury. These anucleate cellular fragments arise from precursor cells called megakaryocytes which are characterized by their large size, polyploid nucleus, and rarity in the bone marrow (1). These cells, in turn, arise from self-renewing pluripotent stem cells which also lead to all other hematopoietic lineages. Megakaryocyte differentiation involves a series of steps of increasing lineage commitment. The end result of this process is the diploid megakaryoblast, which in its continued development, undergoes nuclear endoreduplication, growth in cytoplasmic size, development of α-granules and other granules, and expression of megakaryocyte-specific genes (2, 3). Mature megakaryocytes then release platelets into the peripheral bloodstream (4).

When platelets are activated by agonist such as thrombin, the α-granules release their contents into the surrounding medium. Platelet basic protein (PBP) is one of several proteins released during this process and belongs to the chemokine family of inflammatory mediators. PBP is only present in megakaryocytes and platelets, and represents ~2% of the total platelet protein on a molar basis (5). The biological role of this protein is not completely clear. PBP has been reported to undergo post-translational modifications giving rise to two N-terminally cleaved products, connective tissue-activating peptide III and β-thromboglobulin (β-TG). Additionally, β-TG can undergo further N-terminal cleavage to form neutrophil-activating peptide-2, which is a potent activator of neutrophils (6–8). PBP shares 60% homology to platelet factor 4 (PF4), which is another megakaryocyte-specific protein. These two proteins are expressed at similar times during megakaryocyte development. And while it appears clear that PBP has a role in...
Experimental Procedures

Regulated Expression of the Megakaryocyte-specific PBP Gene

Cloning and Characterization of the Duplicated PBP Gene—A 4.0-kb HindIII fragment containing the entire duplicated PBP gene (previously called β-TGc) was subcloned into similarly digested pBSK vector (Stratagene, La Jolla, CA). Sequencing of this insert was done using the dideoxy chain termination technique with a Sequenase kit (U. S. Biochemical Corp., Cleveland, OH). Universal M13 primers as well as overlapping synthetic primers were used to complete the sequence analysis. Sequence comparison with the PBP gene (previously called β-TG) was performed on a Macintosh Quadra 950 with DNAasias version 2.0 (Hitachi Software Engineering, San Bruno, CA).

Total platelet RNA was extracted from 100 ml of peripheral blood from a normal individual using the guanidinium thiocyanate/acid phenol technique (25). The isolated total platelet RNA was size-fractionated on agarose gels containing formaldehyde and transferred to ZetaBind membrane (Cuno, Meriden, CT). Individual strips were then hybridized to different probes. The probes used were a 0.6-kb PBP cDNA (15), the 185-bp αPbP insert, and the 3.3-kb αIIb cDNA (26), which were all randomly labeled using [γ-32P]dCTP (NEP Life Science Products), calf thymus random primers (Pharmacia Biotech Inc.), and Klenow (Promega, Madison, WI). The filter strips were then washed and exposed to autorradiographic film.

Construction of Reporter Gene Plasmids—The promoter regions of the PBP gene up to 1.4 kb were PCR amplified using VENT DNA polymerase (New England Biolabs, Beverly, MA) as described previously (27). Various sense oligonucleotides were designed to create a series of 5′- truncation promoter constructs. The PBPantisense primer shown below was used for each amplification and is complementary to a portion of the 5′- untranslated region of the PBP gene and has an artificial BgII cutting site near its 5′ end (underlined). The PBPantisense primer is: 5′-GGCCGAGATCTGTTCTCAGAAGCCA-GAAGACC-3′. The various lengths of PCR products were digested with BgIII and then subcloned into Smal-BgII-digested pGL3-Basic vector (Promega) at its polylinker site. All constructs were then sequenced to exclude any PCR-induced mutation. The –92 mutant construct was made by a strategy similar to the 236 mutant that was introduced into the forward oligonucleotide as PBPA1–92, –mut:: 5′-CCACATACCTCCTCATGTCCTCCTTC-3′. The –636 mutant construct was made using the overlapping PCR technique as previously reported (19). The –636 wild-type construct was used as a template for PCR and the two pairs of primers used were the following (the Es sites mutations are underlined): 5′-normal, 5′-CTAAAACCTGCAATGATAAAGGGC-3′ and 5′-mut, 5′-GTGAG-GAAAGGGCAATGGGATGATG-3′; 5′-CATACCTCCTT-GTCTTTCTAC-3′ and 3′-normal, 5′-TACAGGCTTGGAGTAA-AAT-3′. After the second round PCR, the 360-bp amplified fragment was cut with PstI and used to replace the wild-type fragment in –636 plasmid. Restriction enzyme digestion and sequencing were then used to screen for the new construct with the Es site mutation.

The –4500 reporter gene construct was made by replacing the EcorI/KpnI fragment in the –1431 reporter gene plasmid with the 5′-flanking EcoRI/KpnI genomic fragment derived from the full-length PBP gene cloned in pBSK. KpnI is a polylinker site in both of these constructs and the EcoRI site is found within the 5′-flanking genomic sequences.

Transfection of Culture Cells—HEL (28), HeLa, and 293 cells were obtained from American Type Culture Collection (Rockville, MD). They were maintained in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 1% streptomycin/penicillin (Life Technologies, Inc.), 1% t-glutamine (Life Technologies, Inc.) at 37°C in a humidified atmosphere containing 5% CO2. CHRF-288 (29) cells (a generous gift from Dr. Michael Lieberman) were maintained in Iscove’s modified Dulbecco’s medium (Life Technologies, Inc.) supplemented with 20% fetal bovine serum (Life Technologies, Inc.), 1% streptomycin/penicillin (Life Technologies, Inc.), 1% t-glutamine (Life Technologies, Inc.) at 37°C in a humidified atmosphere containing 5% CO2. Twenty-four hours before each transfection, the cells were placed in new medium. Transfection into HEL cells was done using lipofectamine (Life Technologies, Inc.) as suggested by the manufacturer. To standardize for efficiency of transfection, all studies involved cotransfection with pXGH (Nichols Institute Diagnostics, San Juan Capistrano, CA), in which expression of human growth hormone is driven by the mouse metallothionein-1 promoter (30). Various P1 cotransfection studies were done using a P1 expression vector (a generous gift from Dr. Michael Atchison) containing the P1 cDNA under the control of cytomegalovirus promoter (31). For each cotransfection into HEL, 293, and HeLa cells, inflammation, the biological role(s) of PF4 is currently less well defined (9–12).

The gene for the human PBP protein was first cloned in our laboratory. We have demonstrated that this gene is divided into three exons and proceeded 30 bp upstream by a canonical TATA box (13). Our analysis of the physical organization of the PBP gene locus showed that the human PBP gene is immediately upstream of the PF4 gene, facing in the same 5′ → 3′ direction and forming a gene pair occupying ~7 kb. In addition, these two genes are linked within ~1 megabase pairs with other CXC chemokine genes on the long arm of chromosome 4 (14, 15). Furthermore, the PBP and PF4 gene pair is duplicated, and pulsed-field gel analysis suggests that the two pairs of these two genes lie within a region of ~250 kb (16). We have previously shown that the duplicated PF4 expresses a mutant PF4 protein called PF4mut. Northern blot analysis showed that the PF4mut gene is expressed at ~1% of the level of wild-type PF4 (17). The duplicated PBP gene has not been previously characterized.

The molecular basis of megakaryocyte-specific gene expression has been pursued for a number of genes. These studies have defined important regulatory elements and begun to provide insights into how megakaryocyteopoesis is achieved. For example, studies of regulation of megakaryocytic-specific integrin gene αIIb using cell lines and primary cells have localized important GATA and Ets-binding sites in the immediate 5′-flanking region (18–20). Further studies showed that GATA-1 was the particular GATA family member that directly regulated αIIb expression (21). Studies of the rat PF4 promoter using a primary bone marrow expression system have also suggested that a core promoter contains a GATA-binding element at ~31 bp upstream of the transcriptional start site (22). A GATA-1-binding site has also been defined as being important for the regulated expression of the glycoprotein Ibα gene. Mutations of this site leads to absence of expression of this protein in a patient with Bernard-Soulier syndrome (23, 24).

Additional positive and negative regulatory domains have been defined for these genes, but the nature of trans-acting nuclear factors that bind to these sites are not well studied.

Given the fact that only a few megakaryocytic genes have been studied, we decided to examine the molecular basis underlying the expression of the PBP gene to provide another example of how megakaryocyte-specific genes are regulated. In addition, considering the close linkage between PBP and PF4 genes and their coordinated high level expression in developing megakaryocytes, our analysis of PBP gene expression may complement and extend the knowledge already obtained about the regulated expression of the PF4 gene. In this paper, we first demonstrated that the duplicated PBP gene is a non-expressing pseudogene. Next using megakaryocyte-like cell lines, we localized a pyrimidine-rich region in the upstream region of the functional PBP gene which contained a critical positive regulatory element(s) for PBP gene expression. Within this domain, we showed that an Ets-binding element is indispensable for PBP promoter activity. By mobility shift and expression studies, we demonstrated that the protein that bound to this Ets element was the hematopoietic-specific Ets family factor PU.1. Finally, to extend our studies with megakaryocytic cell lines, we also studied PBP gene expression within wild-type or PU.1−/− null embryonic stem (ES) cells which were differentiated into megakaryocytes in vitro. We found that the mouse PBP gene expression level dropped more than 4-fold in PU.1 knockout ES cells compared with wild-type. In contrast to this, the absence of PU.1 expression had little effect on the expression of two other platelet-specific genes, αIIb and PF4.
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1.5 μg of test constructs and 0.5 μg of pXGH vector were used. For transfection studies, 1 μg of expression vector with or without PU.1 coding sequences, 1.5 μg of reporter gene construct and 0.5 μg of pXGH were transfected into HeLa cells.

Transfection of CHRF cells was done by electroporation. Cells grown at 90% confluence were harvested, washed and resuspended in phosphate-buffered saline once. Cells were resuspended in electroporation buffer (30 mM NaCl, 120.7 mM KCl, 8.1 mM NaH2PO4, 1.46 mM KH2PO4, 5 μM MgCl2), 100 μg of plasmid DNA (75 μg of test construct and 25 μg of pXGH) was added to 25 × 10^6 cells in 0.4-cm cuvettes (Life Technologies, Inc.) and then electroporated. The cells were incubated on ice for 15 min and then electroporated using Cell Electroporation System (Life Technologies, Inc.) at 220 V and 800 μF surrounded by ice. After electroporation, cells were allowed to recover 10 min on ice and then 15 min at room temperature. One ml of growth medium was then added to each sample. The samples were spun down and the pellet was resuspended in 3 ml of growth medium. The cells were then grown in six-well 35-mm plates for 2 days at 37°C in 5% CO2.

Forty-eight hours after transfection, cells were lysed and expression levels of reporter genes were assayed. Luciferase activity was measured using β-luciferin on a Lumat LB 9501 luminometer (Berthold, Pittsburgh, PA) as previously reported (33). Human growth hormone activity was determined by radioimmunoassay using a solid-phase two-site radioimmunoassay kit (Nichols Institute Diagnostics) as suggested by the manufacturer. The expression level for each test construct is determined by dividing relative light units by human growth hormone secretion (ng).

Mobility Shift Gel Analysis—Nuclear extracts from HEL, CHRF, and HeLa cells were prepared as described previously (33). Fifty ng of double-stranded oligonucleotide was labeled with [γ-32P]dATP using polynucleotide kinase according to the manufacturer's suggested conditions (Promega). Mobility shift studies were done by incubating 0.1 ng of probe (−10^5 cpm) with 10 μg of nuclear extract or 2 μg of recombinant PU.1 protein in a 20-μl volume containing a final concentration of 20 mM HEPES (pH 7.8), 50–60 mM KCl, 1, 2, 3, or 4 mM MgCl2, 0.5 mM dithiothreitol, 3 μg of poly(dI-dC), 2 μg of salmon sperm DNA (Sigma), 2 μg of bovine serum albumin (F), and 12% glycerol on ice for 20 min. Unlabeled competitor oligonucleotides at various molar excess were added to the nuclear extracts immediately before the addition of the radioactive probe. Reactions were electrophoresed at 12.5 V/cm on a 4% (v/v) polyacrylamide gel in 0.5× TBE (9.9 mM Tris borate, 0.02 M EDTA) buffer in the cold room. The oligonucleotides used in these assays are the following (coding strand): −85/−64, 5′-CCCTCAGCTCTCTCTTCCA-3′; −85/−64M, 5′-CCCTCAGCTCTCTTTTCCA-3′ (mutation underlined); −75/−53, 5′-CTCTTCTCTCTCAGCTCTCTCTCT-3′; EtsM, 5′-TAAACAGACCGACCACTTTTT-3′; Ets, 5′-TAAACACCGACCGACCACTTTTT-3′; K53, 5′-CCCTTAAACAGCCGCGGTGCT-3′; K54, 5′-CCCTTAAACAGCGTCGGCGCT-3′;

The Ets consensus DNA, the Ets mutant DNA, and the SV40 PU.1-binding DNA were designed according to the literature (34, 35). The consensus Sp1-binding DNA were designed according to the literature (34, 35). The primer sets used for PCR amplification are the following: PU.1-S, 5′-GAGGTGGAGACGACTACCCGGAG-3′; PU.1-AS, 5′-TGTGATTGTATCCCTTCGGCGG-3′; HPRT-S, 5′-TACAGGACTAGACACCCTGCTC-3′; HPRT-AS, 5′-GCGGCTGGAAAGAACCTCTT-3′; B1a-S, 5′-CCCTGCGGCGCGGACCCAGCACTTTT-3′; B1b-AS, 5′-CCCTAACCCTTGGAGATGCGCTGCT-3′; B2a-S, 5′-CCCTGCGGCGCGGACCCAGCCAGCAGTTT-3′; B2b-AS, 5′-CCCTAACCCTTGGAGATGCGCTGCT-3′; B3a-S, 5′-CCCTGCGGCGCGGACCCAGCACTTTT-3′; B3b-AS, 5′-CCCTAACCCTTGGAGATGCGCTGCT-3′; B4a-S, 5′-CCCTGCGGCGCGGACCCAGCACTTTT-3′; B4b-AS, 5′-CCCTAACCCTTGGAGATGCGCTGCT-3′; B5a-S, 5′-CCCTGCGGCGCGGACCCAGCACTTTT-3′; B5b-AS, 5′-CCCTAACCCTTGGAGATGCGCTGCT-3′; B6a-S, 5′-CCCTGCGGCGCGGACCCAGCACTTTT-3′; B6b-AS, 5′-CCCTAACCCTTGGAGATGCGCTGCT-3′; B7a-S, 5′-CCCTGCGGCGCGGACCCAGCACTTTT-3′; B7b-AS, 5′-CCCTAACCCTTGGAGATGCGCTGCT-3′.

RESULTS

Regulated Expression of the Megakaryocyte-specific PBP Gene—We started our studies on PBP gene regulation focusing on the immediate 5′-flanking region. The cells were fixed in acetone–methanol (9:1) for 10 min twice, then washed with water and air dried. 1.2 mg/ml rat anti-mouse platelet antibody (a generous gift from Dr. Samuel Burnstein, Oklahoma University, Oklahoma City, OK) was added to each slide and incubated at 37°C, 5% CO2 for 30 min. The slides were then washed with phosphate-buffered saline for 5 min. Then the second antibody fluorescein isothiocyanate conjugate rabbit anti-rat IgG (Sigma) was added onto each slide and incubated for 30 min. The slides were then washed with water and dried in air. Finally the slides were fixed by adding 2 drops of barbital (pH 8.6)/glycerol (1:3) and covered with glass slips. The slides were analyzed using a fluorescent inverted microscope.

Characterization of the 5′-Flanking Region of the PBP Gene—We started our studies on PBP gene regulation focusing on the immediate 5′-flanking region. The cells were fixed in acetone–methanol (9:1) for 10 min twice, then washed with water and air dried. 1.2 mg/ml rat anti-mouse platelet antibody (a generous gift from Dr. Samuel Burnstein, Oklahoma University, Oklahoma City, OK) was added to each slide and incubated at 37°C, 5% CO2 for 30 min. The slides were then washed with phosphate-buffered saline for 5 min. Then the second antibody fluorescein isothiocyanate conjugate rabbit anti-rat IgG (Sigma) was added onto each slide and incubated for 30 min. The slides were then washed with water and dried in air. Finally the slides were fixed by adding 2 drops of barbital (pH 8.6)/glycerol (1:3) and covered with glass slips. The slides were analyzed using a fluorescent inverted microscope.

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cells which are epithelial origin do not (data not shown). In fact, HEL cells have been used by other groups to study regulation of other megakaryocytic genes (18, 21, 32, 34).

HEL and HeLa cells were transiently transfected with a series of reporter gene constructs containing the luciferase cDNA driven by different lengths of the 5′-flanking region of the PBP promoter ranging from 4.5 kb down to 109 bp upstream of the transcriptional start site. Luciferase expression from each test construct was compared with the positive control vector in which the luciferase cDNA is driven by the SV40 promoter/enhancer. The promoterless vector was used as a negative control. The results of this deletional analysis are shown in Fig. 1. In HEL cells, all of the truncation constructs expressed high levels of luciferase activity reaching levels >100-fold above those of the negative control, and comparable to positive control levels (Fig. 1A). Stepwise deletion of the 5′-flanking region of the PBP gene down to −109 bp did not affect reporter gene expression significantly. A similar expression pattern was also obtained using CHRF-288 (29) cells, another megakaryocytic cell line (data not shown). In comparison, transient expression of the identical reporter constructs in non-megakaryocytic HeLa cells resulted in low levels of luciferase activity, which were consistently >50-fold less than the positive control (Fig. 1B). The expression pattern obtained using 293 human fetal kidney cells, which are also non-hematopoietic cells, was identical to that obtained with the HeLa cell line (data not shown). These results suggest that in the transient expression system we used, the 109-bp 5′-flanking region of PBP gene is sufficient to drive high levels of activated expression in megakaryocytic cell lines.

A database search to look for potential binding sites for transcriptional factors within this 109-bp region showed that in addition to the TATA box (boxed in Fig. 2A), this region contains a pyrimidine-rich tract between −85 to −52 bp which includes a number of potential Ets-binding sites (38). In addition, this region contains an inverted core GATA sequence beginning at −94 bp 5′-GTATCA-3′ (in an oval in Fig. 2A), whose 5′-flanking sequence matches the consensus motif for
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FIG. 2. Further analysis of the −109 bp promoter region. A, sequence analysis of the −109 bp PBP promoter region. The transcriptional start site is labeled +1. The TATA element is boxed. The pyrimidine-rich tract is underlined and the PU.1 binding element (see below) is double underlined. The inverted GATA-binding consensus element is in an oval. The probes used in mobility shift studies below, −85/−64 and −75/−53, are indicated. B, further deletion analysis of the inverted GATA site and pyrimidine-rich tract region in HEL cells. The graph is labeled as in Fig. 1, C, similar studies in HeLa cells.

GATA family factor binding sequence (5′-(T/A)(GATA)(A/G)-3′) (39), but the 3′-flanking sequence does not match.

To test whether these potential GATA and Ets sequences are required for PBP gene expression, additional deletion constructs were made and transfected into HEL and HeLa cells (Fig. 2B and C, respectively). In HEL cells, deletion up to −92 bp did not significantly affect reporter gene expression compared with that of −109 bp, suggesting the inverted GATA sequence between nucleotides −97 and −94 is not a functional GATA-binding site. This can be explained by the fact that this GATA sequence does not match the consensus GATA binding motif at its 3′ end (C instead of A/G). However, deletion of the pyrimidine-rich region located between −92 and −50 bp decreased expression by 10-fold, suggesting that this region contains a sequence(s) that was indispensable for PBP promoter activity in vitro (Fig. 2B). In HeLa cells, again, none of these constructs expressed the reporter gene to any significant level (Fig. 2C). The same expression studies were also performed using CHRF-288 and 293 cells which gave similar data as HEL and HeLa cells, respectively (data not shown). Therefore, these data strongly suggest that the sequences between −92 to −50 bp contained binding sites for trans-activating factors that are present in megakaryocytic cells HEL and CHRF-288, but not in HeLa or 293 cells.

Nuclear Factor(s) Binds to the Pyrimidine-rich Region at −85 to −64—As shown in Fig. 2A, the pyrimidine-rich region between −92 and −50 contains several sequences matching the consensus motif 5′-GGAA-3′ for Ets protein binding. To define which if any of these putative Ets sites binds a nuclear factor(s), we performed mobility shift analysis. The DNA probes used to cover this region for mobility shift studies are shown in Fig. 2A. These overlapping double-stranded probes were from base −85 to −64 (−85/−64) and from base −75 to −53 (−75/−53). When incubating the nuclear extract prepared from HEL cells with the −85/−64 probe, several bands were revealed on the gel including a major lower band (Fig. 3A, lanes 1 and 2), the major band is indicated by an arrow (in lane 2). These bands appear to be specific, because they could be competed away by increasing amounts of unlabeled self DNA (Fig. 3A, lanes 3 and 4). In contrast, the −75/−53 probe did not significantly bind to any protein within the HEL cell nuclear extract, suggesting that this region does not contain an intact binding site for any nuclear factor (Fig. 3A, lanes 5 and 6). To determine whether the protein(s) bound to the −85/−64 probe is specific only to the HEL cell line, we incubated this probe with nuclear extracts prepared from CHRF-288 and HeLa cells (Fig. 3B, lanes 11–13 and 14–16, respectively). On mobility shift gel, the upper bands seen with HEL nuclear extract were also present when using HeLa and CHRF-288 nuclear extract. In addition, CHRF-288 nuclear extract had the same major lower band as with HEL cells, while HeLa cells did not show this band, but had a major band at a slightly higher position. Since both HEL and CHRF-288 cells are megakaryocytic cell lines, while HeLa is a non-hematopoietic cell line, these findings suggest that there may be a tissue-specific protein(s) in HEL and CHRF-288 cells but not HeLa cells involved in the formation of the fast migrating band. It is possible that the restricted expression pattern of this factor may be responsible for the high level expression seen with the truncation constructs in HEL and CHRF-288 cells but not in HeLa or 293 cells. We, therefore, focused our studies on characterizing the protein(s) involved in this band formation using HEL cell nuclear extracts.

Identification of an Ets-binding Site between −85 and −64 Region—To determine the protein(s) that binds to the −85/−64 probe, we needed to pinpoint the exact site of protein binding. To accomplish this, we tested whether the major complex could be competed away by the overlapping −75/−53 fragment. Two hundred-fold excess of cold −75/−53 DNA could not compete away formation of this fast-moving complex, suggesting that the overlapping region of these two probes was not involved in binding (Fig. 4A, lane 6). Close inspection of the sequences covered in the oligonucleotide −85/−64 revealed an inverted Ets-binding motif (5′-TTCC-3′) between −78 to −75 bp, immediately upstream to the −75/−53 DNA. To test whether this Ets sequence is the site for protein binding, we tried to compete away the major complex with cold −85/−64 wild-type oligonucleotide or a mutated one with a 5′-TTCC-3′ to 5′-TTGG-3′ conversion at Ets site −78 to −75. Indeed, the mutated −85/−64 oligonucleotide lost the ability to compete away the major complex which bound to the wild-type probe (Fig. 4A, lane 5).
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Critical for PBP Promoter Activity— PU.1 Binds to the Ets Site between −78 to −75—Next, we asked which Ets protein bound to the Ets sequence at −78 to −75 in front of the PBP gene? This flanking sequence of the −78 to −75 inverted Ets site is an inverted 5′-GAGGAA-3′ site, known as a PU box which is specifically recognized by the Ets family transcription factor called PU.1 (40). In addition, the fast mobility of the major lower band seen on the gels in Fig. 4 suggested that the protein involved in this complex might be of low molecular weight. These analysis makes the Ets family member PU.1 a good candidate. PU.1 has a low molecular mass of 35 kDa when compared with Ets-1 and Ets-2, which have molecular masses of 54 kDa (41). PU.1 has also been shown to be expressed only in hematopoietic lineages, including megakaryocytes and megakaryocytic cell lines such as HEL (42).

To test whether PU.1 binds the −78 to −75 bp Ets site, we used a sequence from the SV40 promoter (5′-TAACCTCT-GAAAGGAGAACATTGGT-3′) (35), which has been shown to specifically bind PU.1, as a cold competitor in our mobility shift studies. This DNA was shown to be an effective competitor for the formation of the fast migrating complex, suggesting that PU.1 was involved in this complex (Fig. 4C, lanes 18 and 19). We then asked whether recombinant PU.1 protein could bind to the PU box in the 5′-flanking region of the PBP gene. Incubation of the −85/−64 probe with in vitro translated recombinant PU.1 protein resulted in the formation of a complex running at the same mobility as the fast migrating band with HEL cell nuclear extract (Fig. 5A, lane 4). The fainter and faster mobility band below the major complex resulted from internal initiation during translation of the PU.1 mRNA in vitro, giving a smaller PU.1 product (35). These results imply that binding of PU.1 to its cognate site at −78 to −75 is not associated with any other proteins. To further confirm that the fast migrating complex seen with the PBP −85/−64 probe contains PU.1, we performed supershift studies using rabbit polyclonal anti-human PU.1 antibody. This antibody recognizes the DNA-binding domain of PU.1 and therefore, abolishes its binding to the cognate site. Incubating the −85/−64 probe, HEL nuclear extract mixture with increasing amounts of anti-PU.1 antibody prior to electrophoresis resulted in sequential abolishment of the fast migrating band (Fig. 5B, lanes 7–9). On the other hand, incubation with a non-related anti-human Sp1 antibody at the same concentrations did not have any effect on complex formation (Fig. 5B, lane 10). Conversely, this anti-PU.1 antibody, unlike the anti-Sp1 antibody, failed to supershift Sp1 bound to a consensus Sp1 probe (Fig. 5B, lanes 11–13). Thus, these data indicate that the transcription factor PU.1 alone binds to the Ets consensus sequence at −78 to −75 bp in the immediate 5′-flanking region of PBP gene.

Binding of PU.1 to Its Cognate Site at −78 to −75 bp Is Critical for PBP Promoter Activity—To understand the biological significance of PU.1 binding to its cognate site at −78 to −75 bp, we introduced a CC to GG conversion at this site into

compared with lanes 3 and 4). To confirm that this is an Ets site, we also used an Ets consensus DNA (5′-ATAAACAG-GAAGTGTT-3′) as a cold competitor. Again, excess amounts of this consensus DNA specifically competed away the fast-mov-
two luciferase reporter gene constructs, one containing 92 bp and the other 636 bp of the immediate PBP 5'-flanking region. This mutation is identical to that which prevented the -85/-64 DNA from binding to PU.1 (Fig. 4A, lane 5). Transient expression studies with mutant reporter gene constructs in HEL cells showed that this mutation caused a 5-fold decrease in expression for the 92-bp construct, and an 8-fold decrease of expression for the 636-bp construct relative to the wild-type constructs (Fig. 6A). The same results were seen in CHRF-288 cells (data not shown). In HeLa cells, expression of these mu-

Fig. 4. Mobility shift studies with different cold competitors. A, mobility shift studies with the wild-type -85/-64 probe and competition with cold wild-type and mutated -85/-64 DNA or the -75/-53 DNA. The latter two were at 200:1 molar excess. The triangle refers to a 50:1 and 200:1 excess. B, mobility shift studies with the wild-type -85/-64 probe and competition with cold wild-type -85/-64 DNA, wild-type, or mutated Ets consensus DNA, all at a 200:1 excess. In lanes 12 and 13, mobility shift studies using the Ets consensus DNA probe with or without HEL nuclear extract, demonstrating that a series of bands are observed, one of which has the same mobility as the fast migrating band seen with -85/-64 probe. C, mobility shift studies with the wild-type -85/-64 probe and competition with cold wild-type -85/-64 DNA or the PU.1 binding sequence from the SV40 promoter. The closed triangle again refers to competition with 50:1 and 200:1 molar excess. In all three figures, the arrow points to the fast migrating band of interest.
tant vectors remained as low as the wild-type constructs (data not shown). These findings suggest that the PU.1-binding site is critical for high level expression of the PBP gene in megakaryocytic cell lines.

We then asked whether exogenous PU.1 expression would be sufficient to lead to significant promoter activity from the PBP promoter. We performed transactivation experiments by co-transfecting HeLa cells which do not express PU.1 (43) with a cytomegalovirus promoter-driven PU.1 expression vector along with luciferase reporter constructs containing either wild-type or mutant PBP promoter. In control experiments, the same cytomegalovirus-driven expression vectors not containing the PU.1 coding region were co-transfected. As shown in Fig. 6B, luciferase expression from the 92-bp PBP promoter construct was stimulated 8.7-fold in the presence of exogenously expressed PU.1. On the other hand, only a 2.9-fold increase in expression was seen in transactivation experiments when the PU.1-binding site was mutated. Similarly, co-expression of PU.1 increased expression of the 636-bp PBP promoter construct 5.2-fold, while mutation of the PU.1-binding site had only a 2.1-fold increase in expression. Transactivation also occurred from truncation constructs having longer 5'-flanking regions. These findings indicate that binding of PU.1 to its
cognate site in the immediate 5′-flanking region of the PBP gene is important for this gene’s promoter activity in vitro, and its supplement can lead to significantly increased promoter activity in a non-hematopoietic cell line.

**PU.1 Is Required for PBP Gene Expression in Megakaryocytes Differentiated from ES Cells**—Previous in vitro studies have demonstrated that PU.1 is a critical transcriptional regulator in several hematopoietic tissues. Our data shown here is the first to implicate that PU.1 is critical for expression of a megakaryocytic gene PBP. The studies described above in HEL and CHRF-288 cells, however, rely on a transient expression system using reporter plasmids and do not account for the possible influences of the chromatin which surrounds the PBP gene locus or any cis-regulatory regions outside of the immediate 5′-flanking region of the gene. Furthermore, although HEL cells are megakaryocytic in nature they also have erythroid and monocytic features (44, 45). These facts prompted us to examine the role that PU.1 plays in PBP gene expression in its natural megakaryocyte cellular environment and chromatin context.

To accomplish this, we have utilized the in vitro differentiation of the ES cells system. During in vitro differentiation, ES cells form three-dimensional structures, known as EBs, which contain a variety of embryonic cells, including hematopoietic tissues. When differentiated in 0.9% methylcellulose medium, EBs can give rise to primitive and definitive erythrocytes, macrophages, rare neutrophils, or megakaryocytes depending on which exogenous growth factors they are exposed to (46, 47). In our experiments, we differentiated wild-type and PU.1−/− knockout CCE ES cells (36) in 0.9% of methylcellulose medium in the presence of various cytokines including interleukin-3, interleukin-6, stem cell factor plus thrombopoietin, which has been shown to be necessary for megakaryocyte differentiation (48). EBs started forming at day 4 from both PU.1+/+ wild-type and PU.1−/− null ES cells. We harvested these EBs on day 13 according to published procedures (36). In addition, we disaggregated some EBs and replated them in methylcellulose medium. Single colonies formed 4 days later. Microscopic examination of the cells within these colonies from both PU.1+/+ and PU.1−/− lines revealed that they included colonies morphologically similar to megakaryocytes, being large and polyploid, and stained positive with a rat anti-mouse platelet antibody (data not shown). Semi-quantitative RT-PCR assays were carried out using total RNA isolated from day 3 EBs, and γ32P-labeled primer pairs specific for murine PBP and two other megakaryocytic genes, PF4 and αIib. As a control, murine PU.1 and ubiquitous HPRT messages were also amplified. Fig. 7A shows the autoradiograph of PCR products on the gel. The expression levels of the five tested genes measured in the linear range of PCR amplification from both wild-type and PU.1−/− knockout ES cells are summarized in Fig. 7B. As expected, PU.1 is only expressed in wild-type differentiated ES cells. The ubiquitous gene HPRT is expressed equally in both wild-type and PU.1−/− knockout ES cells. However, the PU.1−/− null ES cells had a markedly decreased level of expression of the PBP gene. While αIib is expressed at normal levels 104.5 ± 4.7% in the PU.1−/− knockout ES cells and PF4 is expressed at 67.9 ± 17.8% of the wild-type level, PBP gene expression decreased to 22.2 ± 1.7% of the PU.1+/+ wild-type cells. These results are consistent with our transient expression studies and further confirm that PU.1 is a critical trans-acting factor for PBP gene expression.

**DISCUSSION**

Tissue-specific expression of a gene often occurs at the transcriptional level and may simply require the binding of a tissue specifically expressed factor to the regulatory element of the gene or may need a unique combination of several factors (49, 50). Megakaryocytopenia is the process by which the multipotential stem cells in bone marrow differentiate into mature megakaryocytes. It is characterized by significant morphological changes as well as the expression of megakaryocyte-specific genes (51–53). Given the fact that this process is not completely understood and only a few megakaryocytic genes have been studied in detail, our studies on the regulation of PBP gene, which is exclusively expressed in megakaryocytes should contribute to the understanding of mechanisms controlling gene expression in this hematopoietic lineage.

In this paper, we examined the 5′-flanking region of the megakaryocyte-specific PBP gene initially using an in vitro transient expression system. We found that a minimum of 109 bp of upstream sequences was enough for promoter activity in megakaryocytic cell lines. Within this region we identified a putative binding site for the hematopoietic transcription factor PU.1. By conducting mobility shift analysis and expression studies, we confirmed that PU.1 bound to its cognate site in this region and was necessary for PBP promoter activity.

The biological role of PU.1 in PBP regulation was further confirmed by our analysis of its expression level in PU.1+/+ wild-type and PU.1−/− null ES cells which were differentiated in the presence of thrombopoietin along with other growth factors. These cells resembled megakaryocytes both morphologically and immunologically. Furthermore, these cells express several megakaryocyte-specific genes. Results from these
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At the moment, the explanation of how normal megakaryocytopoiesis can proceed with the absence of important nuclear factors such as GATA-1 and PU.1 is unclear. One possibility is that while GATA-1 and PU.1 are important for certain megakaryocyte genes to be expressed, they are not necessary for the survival and development of the megakaryocyte precursor. Alternatively, redundancy and overlapping function of transcription factors within a family may explain the continued megakaryocytopoiesis. For GATA-1, it may be that the coincident expression of another GATA family member, GATA-2, can substitute for GATA-1, allowing megakaryocytopoiesis to occur (68, 69). Indeed, increased GATA-2 expression was detected in erythroid cells cultured from GATA-1-null ES cells (70). If this model is right, it would suggest that there is another Es family member in the developing megakaryocytes that may be able to substitute for PU.1 and that this redundancy protects megakaryocytopoiesis in the PU.1 knockout animal. Therefore, the possibility exists that other megakaryocyte-specific genes depend on PU.1 binding for their megakaryocyte-specific expression, and further studies of other megakaryocyte-specific genes and the role of PU.1 in their regulated expression need to be examined.

Acknowledgments—We thank Suchandra Majumdar, Chengqing Li, and Qing He for technical assistance in the early stages of this effort. We also thank Dr. Samuel Burnstein for providing the rat anti-mouse polyclonal anti-platelet antibody.

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studies showed that PBP expression decreased significantly in cells not expressing PU.1. This corroborates the results from our in vitro studies using megakaryocytic cell lines.

PU.1 was first discovered as an oncogene leading to the development of several virus-induced tumors (54, 55). This Ets family member has been shown to be normally expressed only in hematopoietic tissues, predominantly in B lymphocytic, granulocytic, and mononuclear cells (42, 43). In accordance with its specific expression pattern, it has been shown to function as a major transcription activator in the expression of lineage-specific genes within these cells (56). For example, it has been shown that PU.1 is able to recruit and interact with another nuclear factor NF-EM5 at the immunoglobulin κ 3′-enhancer region, thereby promoting expression (35). The myeloid cell-specific c-fes promoter is regulated by Sp1, PU.1, and an unidentified transcription factor. Furthermore, PU.1 can transactivate the c-fes promoter in non-myeloid cell lines (57). The granulocyte colony-stimulating factor receptor is expressed exclusively in myeloid cells and platelets (58, 59). Expression studies show that the 1391-bp fragment of the granulocyte colony-stimulating factor receptor promoter is both active in myeloid cell lines and tissue-specific. Two PU.1-binding sites are localized at +36 and +43 of 5′-untranslated region and are shown to be important for the promoter activity since mutation of them reduces promoter activity to 75% (60).

Targeted disruption of the PU.1 gene in mice produces a late gestation lethal mutation that appears to result in a major disruption of the myeloid and lymphoid lineages (61). This can be explained by the fact that some of the genes regulated by PU.1, such as CD11b, CD18, and granulocyte colony-stimulating factor receptor have been shown to play important roles in PU.1, such as CD11b, CD18, and granulocyte colony-stimulating factor receptor have been shown to play important roles in its specific expression pattern, it has been shown to function as a major transcription activator in the expression of lineage-specific genes as described for the PBP gene in PU.1−/− null ES cells remains unknown.

At the moment, the explanation of how normal megakaryocytopoiesis can proceed with the absence of important nuclear factors such as GATA-1 and PU.1 is unclear. One possibility is that while GATA-1 and PU.1 are important for certain megakaryocyte genes to be expressed, they are not necessary for the survival and development of the megakaryocyte precursor. Alternatively, redundancy and overlapping function of transcription factors within a family may explain the continued megakaryocytopoiesis. For GATA-1, it may be that the coincident expression of another GATA family member, GATA-2, can substitute for GATA-1, allowing megakaryocytopoiesis to occur (68, 69). Indeed, increased GATA-2 expression was detected in erythroid cells cultured from GATA-1-null ES cells (70). If this model is right, it would suggest that there is another Ets family member in the developing megakaryocytes that may be able to substitute for PU.1 and that this redundancy protects megakaryocytopoiesis in the PU.1 knockout animal. Therefore, the possibility exists that other megakaryocyte-specific genes depend on PU.1 binding for their megakaryocyte-specific expression, and further studies of other megakaryocyte-specific genes and the role of PU.1 in their regulated expression need to be examined.
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