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## Bactericidal/Permeability-Increasing Protein (BPI) and Proteinase 3: Studies at the Transcriptional Level

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## AML-1, PU.1, and Sp3 regulate expression of human bactericidal/permeability-increasing protein

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### Abstract

Bactericidal/permeability-increasing protein (BPI) is an antimicrobial protein in neutrophils, stored in azurophil granules. Expression of BPI is absent in neutrophils of newborns and patients with secondary granule deficiency (SGD), possibly contributing to dysfunction of neutrophils. We report two alternative transcription start sites at 52 and 22 bp upstream of the translation start. A proximal 222 bp promoter conferring expression in myeloid cells was identified, and critical *cis*-acting sites for myeloid expression were contained within the 159 bp upstream of translation start. Within this region, direct binding and transactivation by AML-1, PU.1, and Sp3 were demonstrated, as judged by electrophoretic mobility shift analysis. Moreover, transient transfections of C/EBP $\alpha$  or C/EBP $\beta$  to HeLa cells resulted in increased promoter activity, indicating a direct or indirect role for C/EBP. In conclusion, we provide evidence for AML-1, PU.1, and Sp3 cooperatively and directly mediating BPI-expression during myeloid differentiation. © 2003 Elsevier Inc. All rights reserved.

**Keywords:** Transcription; Promoter; Gene; Transcription factor; Myeloid; Azurophil granule protein; Bactericidal/permeability increasing protein

The neutrophil granulocyte is a major effector cell of the innate defence against invading microbes. Neutrophils are characterised by a large number of cytoplasmic granules containing proteins and peptides necessary for the specialised functions of the cell. Among these organelles are the azurophil (primary) granules. Several antimicrobial proteins such as myeloperoxidase, serine proteases, defensins, and the bactericidal/permeability increasing protein (BPI) dominate the content of the azurophils (reviewed in [1,2]). BPI was initially identified as an antibacterial protein in neutrophils [3] where it is a major constituent (up to 1% of total protein) [4], but is also present in granules of human eosinophils [5] and on the surface of monocytes [6]. Recently, it was also shown that epithelial cells synthesise BPI [7]. BPI is a 55 kDa cationic protein with cytotoxic and opsonic activities against Gram-negative bacteria (reviewed in [8]). Initial binding of BPI to live bacteria results in a reversible increase of outer membrane permeability and arrested

cell division. Killing of the bacteria follows, correlating to inner membrane damage [9,10]. Direct binding of BPI to the bacterial envelope is critical for its antimicrobial action and BPI shows high affinity to the lipid A moiety of lipopolysaccharide (LPS) of Gram-negative bacteria [11]. The structural determinants for the LPS-interaction are located solely in the amino-terminal half of the protein, while an opsonic function of BPI is also dependent on the carboxy-terminal half of the protein [12] (reviewed in [8]). Consistent with its high affinity for LPS, BPI is a paralogue to the acute phase protein lipopolysaccharide-binding protein (LBP) (reviewed in [13]). LBP mediates LPS-binding to inflammatory cells, thus evoking a strong inflammatory response with increased secretion of proinflammatory cytokines, e.g., tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin 1 (IL-1). When secreted excessively, these may give rise to septic shock with multi-organ failure (reviewed in [14]). In contrast, BPI neutralises the pro-inflammatory effects of LPS [15] and BPI could be of potential clinical use in the treatment of fulminant Gram-negative infections [16].

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BPI is stored together with a wide range of other bactericidal proteins and antimicrobial polypeptides, e.g., serine proteases and defensins in the azurophil granules. Formation of neutrophil granules occurs sequentially during maturation; azurophil granules are the first to appear during the promyelocytic stage followed by specific granules in myelocytes. Segregation of different proteins into distinct granule populations is achieved by temporal separation of the synthesis of the granule content [2]. Thus, proteins stored in azurophil granules are synthesised prior to those of specific granules. However, the expression of different azurophil proteins is not completely synchronised, e.g., the levels of mRNA for defensins do accumulate to their highest level after the peak of mRNA for most other azurophil proteins [17]. Consistently, the biosynthesis of defensin protein occurs later than that of most other azurophil proteins [17], which may explain the enrichment of defensins in a subset of large azurophil granules [18]. BPI has a uniform peripheral distribution in all subtypes of azurophil granules [19]. Interestingly azurophil granules in the newborn are specifically deficient in BPI, whilst still being equipped with normal amounts of other azurophil granule proteins such as myeloperoxidase and defensins [20,21], arguing for a distinct regulation of BPI-gene expression. The transcriptional control of BPI has not been investigated, but in some cases of specific granule deficiency (SGD), a rare disease with absent gene expression of several proteins stored in specific granules, BPI is also affected. Recently, SGD was linked to homozygous elimination of the neutrophil transcription factor C/EBP $\epsilon$  [22]. Elimination of C/EBP $\epsilon$  in mice resulted in a phenotype resembling human SGD [23]. However, since murine neutrophils do not contain BPI [24] it is not possible to determine the effects on BPI in this animal model.

Thus, previous observations indicate that the transcriptional control of BPI in some circumstances may be separate to that of the genes coding for other azurophil granule proteins such as myeloperoxidase. The aim of this work was therefore to functionally characterise the proximal promoter of human BPI in myeloid cells. We report a proximal promoter, consisting of the initial 222 bp upstream of the translation start, conferring myeloid-specific expression. We show that AML-1, PU.1, and Sp3 bind directly and activate BPI-expression. This is the first report of Sp3 in the transactivation of a gene encoding a neutrophil azurophil granule protein.

## Materials and methods

**Cell culture.** U937-4, a subclone of U937 [25], HeLa, K562, and HL60 cells were maintained in RPMI-1640 medium (GIBCO) supplemented with 10% fetal calf serum (FCS, GIBCO). HL60 clone 15 (ATCC No. CRL-1964) was maintained in RPMI-1640 with 10% FCS supplemented with L-glutamine (20 mM), sodium pyruvate (1 mM), and glucose (4.5 g/l). Normal mononuclear bone marrow cells were obtained from healthy donors after informed consent.

**Reverse transcription PCR.** Reverse transcription PCR (RT-PCR) of 1  $\mu$ g total RNA was performed using the GeneAmp RNA PCR-kit (Perkin Elmer). The primer 5'-TGGAGGTGCCACAGACCA TAGTT-3' was used for reverse transcription and also in the subsequent 35 cycle PCR, together with the upstream primer 5'-ATGAGAGAGAACATGGCCAGGGGC-3'.

**Northern blot.** Northern blot was performed with the Express-Hybridisation solution (Clontech). Fifteen  $\mu$ g total RNA was separated on a formaldehyde gel followed by transfer to a Genescreen plus membrane (NEN). Equal loading and transfer efficiency were checked by Radiant red RNA-stain (BioRad). A 730 bp cDNA, corresponding to the amino-terminal half of BPI, was labelled with [<sup>32</sup>P]dCTP using the Rediprime II kit (Amersham-Pharmacia Biotech). High stringency hybridisation was performed at 68 °C. The membrane was washed with 2 $\times$  SSC, 0.05% SDS 3 $\times$  10 min at room temperature and 0.1 $\times$  SSC, 0.1% SDS 2 $\times$  20 min at 50 °C. Membranes were analysed using a Molecular Imaging FX analyser (BioRad).

**Genomic cloning.** Cloning of genomic DNA upstream of the translation start was performed by PCR utilising the human Genomewalker-kit (Clontech). From the published cDNA sequence [26], oligonucleotide downstream primers, specific for BPI, were designed and used in nested PCR. The adapter primer was 5'-TGTCACGGCGGTGCCTATGG CGA-3' and nested primer 5'-TCGAAGATCTTCTCATCTCCA GAGCTGCCAAAACCTC-3' (*Bgl*II restriction site underlined). PCR product was cloned into pGL3/basic vector (Promega) and sequenced on both strands.

**5'-rapid amplification of cDNA-ends.** Total RNA was isolated from normal human mononuclear bone marrow cells or mRNA from the human promyelocytic cell line HL60. The 5'-end of the mRNA (transcription start) was identified by 5'-rapid amplification of cDNA-ends (RACE) using the 5/3' Race kit (Roche) or the First choice RLM-race kit (Ambion). Nested PCR in the RACE reactions was performed with adapter primer 5'-GACCCAGCAGCCAGGGTTGAC-3' and nested primer 5'-GACGAGCACCATCAGGGACAC-3', both binding to cDNA downstream of the translation start. RACE-products were cloned into TOPO TA cloning plasmids (Invitrogen) and sequenced.

**Deletion and site-directed mutation constructs.** To create sequential 5'-deletions of the promoter region, PCR was performed with the cloned genomic DNA as template. The nested primer described above was used as the downstream primer, together with upstream primers complementary to sequences in the promoter at -897, -693, and -222 bp, respectively. Numeration according to the translation start as #1 is used here and throughout this work. PCR products were cloned into pGL3/basic to create the reporter vectors pGL3/897, pGL3/693, and pGL3/222, respectively. The further extended deletions were created with the Erase-a-base System (Promega). For site-directed mutagenesis of potential transcription factor binding sites, oligonucleotide primers including the desired mutations were synthesised and used in two-step splice overhang extension (SOE) PCR as described [27]. The following potential transcription binding sites were mutated: **C/EBP-site** at position -100 bp, 5'-CTTTC-3' changed to 5'-AGGGA-3'; **C/EBP-site** at position -77, 5'-CATT-3' changed to 5'-ACGG-3'; **AML-1-site** at -155 bp, 5'-ACCAC-3' changed to 5'-CAACA-3'; **AML-1-site** at -142 bp, 5'-ACCAC-3' changed to 5'-CAACA-3'; **Sp1/Sp3-site** at -136 bp, 5'-GCCCT-3' changed to 5'-TAAAG-3'; and **MZF-1-site** at 69 bp, 5'-CCCAG-3' changed to 5'-AAACA-3'. In all constructs only one binding site was mutated, with the exception of pGL3/222AML1/D in which both sites (-155 and -142) were modified and pGL3/222C/EBP/D in which both C/EBP-sites (-100 and -77) were mutated. After subcloning into pGL3/basic, mutations were verified by sequencing.

**Transient transfections.** For the luciferase assays, electroporation was performed. The transfection conditions for each cell line were optimised, as judged by maximal luciferase activity, with regard to cell density, voltage, DNA concentration, and incubation time between transfection and analysis. pGL3 plasmid was mixed with 0.5  $\mu$ g pRL-SV40 vector used as internal control for transfection efficiency (all plasmids from Promega) and added to cells in 0.5 ml of culture

-156/-121 bp AACCAATGGAAAACACAGCGGCCCTCCCTCTTT  
 -156/-121 bp ACAACAAATGGAAAACAACAAGCGGCCCTCCCTCTTT AML1 mut.  
 -156/-121 bp AACCAATGGAAAACACAGCGTAAAGCCCTCTTT unspc mut.  
 -149/-108 bp ATGGAAAACACAGCGGCCCTCCCTCTTTCCACCCCTACCA  
 -149/-108 bp ATGGAAAACACAGCGTAAAGCCCTCTTTCCACCCCTACCA Sp1/Sp3 mut.  
 -107/-76 bp GGTTCCTCTCTTCCACATCTACTGACT  
 -107/-76 bp GGTTCCTCTCTCAGGGACATCTACTGACT C/EBP mut.

Fig. 1. Oligonucleotide probes for EMSA. Numeration according to Fig. 5. The site-directed mutations are underlined.

medium. Electroporation was performed in a 0.4 cm cuvette (Gene-pulser II, BioRad) at a capacitance of 960  $\mu$ F. After electroporation, cells were seeded in 10 ml of culture medium and incubated at 37 °C for the given time period, after which dual luciferase assay (Promega) was performed. The amount of pGL3 plasmid, electrical settings, cell number during electroporation, and time of culture prior to luciferase assay for the different cell lines were as follows: HL60, 35  $\mu$ g DNA/340 V/5  $\times 10^6$  cells/10 h; HeLa, 15  $\mu$ g DNA/300 V/5  $\times 10^6$  cells/16 h; U937, 15  $\mu$ g DNA/280 V/8  $\times 10^6$  cells/16 h; and K562, 15  $\mu$ g DNA/280 V/8  $\times 10^6$  cells/16 h. The pGL3/promoter vector containing a SV40-promoter, and the pGL3/basic vector lacking promoter, were used as positive and negative controls, respectively.

For transient overexpression of C/EBP $\alpha$ , C/EBP $\epsilon$ , PU.1, AML1-ETO, and AML1b, expression vectors were used (vectors were kindly provided by Dr. Pierre A hman, Gothenburg University, Sweden, Dr. Phil Koeffler, UCLA, Los Angeles, USA, Dr. Mikael Sigvardsson, Lund University, Lund, Sweden, and Dr. Scott Hiebert, St. Jude Children's Reserve Hospital, Memphis, USA, respectively). HeLa cells were transfected with Lipofectin reagent (Invitrogen Life technologies).

**Luciferase assay.** Cell lysis and the luciferase assay were performed using the Dual luciferase reporter assay-kit (Promega). The light emission of firefly and *Renilla* luciferase in lysate supernatant was quantified in a TD-20/20 luminometer (Turner Design). The values of firefly luciferase were normalised to values of *Renilla* luciferase, which was used as an internal control for transfection efficiency.

**Protein extracts and electrophoretic mobility shift assay (EMSA).** Nuclear extracts were prepared as previously described [28], with the modification that 0.6% NP-40 was included in the lysis buffer and protease inhibitors (Complete, Roche) were used in all buffers. Probe was prepared by labelling single-stranded DNA oligonucleotide with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase (Roche). Labelled oligonucleotide was purified from free isotope on MicroSpin G-25 columns (Amersham-Pharmacia Biotech) and thereafter annealed to the corresponding complementary oligonucleotide. Nuclear extract (4–6  $\mu$ g) was incubated with labelled DNA probe for 25 min at room temperature, in binding buffer (5 mM HEPES, pH 7.9, 25 mM KCl, 1.25 mM MgCl<sub>2</sub>, 0.5 mM DTT, 0.5 mM EDTA, 1 mM ZnCl<sub>2</sub>, and 50% glycerol) supplemented with 0.3–0.5  $\mu$ g poly(dIdC)/15  $\mu$ l reaction mixture (Amersham-Pharmacia). Unlabelled competitor probe was added together with labelled probe, while the antibodies (all from Santa Cruz, Biotechnology) were added after 20 min of incubation followed by 10 min of further incubation at room temperature. The samples were separated on 6% polyacrylamide TBE gel and analysed on a Molecular Imaging FX analyser (BioRad). The oligonucleotides used for EMSA were as shown in Fig. 1.

## Results

### Expression of the BPI gene in haematopoietic cells

Different haematopoietic cell lines and one epithelial carcinoma cell line were screened by Northern blotting

for expression of BPI transcript. As shown in Fig. 2, NB4, U937, K562 as well as HeLa cells were negative for the expression of BPI. However, both HL60 clones analysed expressed BPI at a level comparable to that of fresh bone marrow cells (Fig. 2). Equal loading of RNA was verified by Radiant Red staining (not shown). Similar results were seen with RT-PCR (data not shown).

### Identification of the transcription start using 5'-RACE

Total RNA extracted from human bone marrow from two healthy individuals, and mRNA extracted from HL60 mononuclear cells, was subjected to 5'-RACE. In repeated 5'-RACE reactions, a PCR-product of approximately 180 bp was detected. After cloning, 10 individual clones from five separate PCRs were sequenced. The cDNA sequence upstream from the translation start could be continuously aligned to genomic sequence, demonstrating that no previously unidentified intron upstream of exon 1 exists. Out of 10 sequenced clones, 3 clones terminated on base pair –52 in regard to the translation start and 7 clones terminated at base pair –22 (Fig. 3). We conclude that two alternative transcription start sites are utilised at –22 and –52, respectively.

### Cloning of the BPI-promoter

Two PCR products obtained from the amplification of genomic DNA of 1.1 and 2.2 kb were cloned into pGL3/basic vector (Promega). Sequencing revealed that both PCR products, as expected, contained sequences corresponding to BPI-cDNA. The sequence upstream of

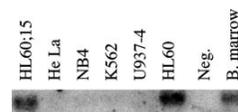


Fig. 2. Expression of BPI in bone marrow cells and cell lines. Total RNA was extracted and subjected to Northern blotting and high stringency hybridisation with a cDNA of BPI. Expression of BPI is seen in normal bone marrow progenitor cells, as well as in both HL-60 clones analysed. Myeloid U937, K562, NB4, and epithelial HeLa cells show no detectable level of BPI-expression. No RNA is used as negative control.



Fig. 3. Two alternative transcriptional start sites. After RNA isolation of normal bone marrow cells or HL-60 cells, 5'-RACE was performed. Two alternative transcription start sites at  $-52$  and  $-22$  bp, respectively, are indicated with arrows. The sequence of cloned mRNA is aligned to the genomic sequence. Numeration here, and throughout this work, starts from the first nucleotide upstream of the translation start codon ATG as number  $-1$ .

the cDNA sequence was identical in both PCR products. Further sequencing was therefore confined to the 2.2 kb product. The obtained sequence revealed complete identity to the genomic sequence from chromosome 20, which recently became retrievable from GenBank (#NT 011362).

#### Defining the proximal regulating promoter

To localise *cis*-acting regions of the promoter important for driving the expression of BPI in myeloid cells, we performed transient transfections of pGL3/1.1 and pGL3/2.2 into HL60 clone 15 (Fig. 4A) and HL60 cells (data not shown). Both clones showed endogenous BPI-expression (Fig. 2). While pGL3/2.2 showed significant promoter activity, as measured by luciferase activity, the expression of pGL3/1.1 resulted in more than twice the luciferase activity (Fig. 4A). When matching the 1.1 kb promoter sequence to the transcription factor database TRANSFAC [29] with various search programs [30,31], some potential *cis*-acting elements were found, including both potential activator and repressor-sites. We therefore chose to continue creating progressive 5'-deletions. PCR-products were cloned into pGL3, creating pGL3/897, pGL3/693, and pGL3/222 plasmids. Upon transfection to HL60 clone 15 cells, pGL3/897 and pGL3/693 showed an increase in the capacity to drive expression of the luciferase reporter, as compared to pGL3/1.1. Notably, a significantly stronger transcriptional activity was seen with pGL3/222. We therefore conclude that the region 222 bp upstream of the translation start contains the proximal promoter, including critical regulatory *cis*-elements responsible for the expression of the BPI-gene.

To correlate the activity of the promoter to the level of expression of the endogenous BPI-gene, the reporter construct of the proximal promoter pGL3/222 was transfected to three other human haematopoietic cell lines and one epithelial carcinoma cell line; the erythropoietic K562, the monoblastic U937-4, the promyelocytic NB4, and the epithelial HeLa cell line, all having a BPI-expression below the level of detection (Fig. 2). In all three haematopoietic cell lines, a low but significant activity of the BPI-promoter was demonstrated (Fig. 4B). Thus, although the promoter activity is very high in BPI-expressing HL60 clone 15 cells, as related to the level of transcription from the control vector pGL3/

promoter, the proximal promoter is also active in myeloid cells with no or very low expression of BPI. Promoter activity was, however, restricted to myeloid cells, since transfection into non-myeloid human epithelial carcinoma HeLa cells resulted in luciferase activity that was not above background levels (Fig. 4B).

To further map the proximal promoter with respect to regulatory *cis*-elements, we made extended deletion-constructions that were transfected into HL60 clone 15. Critical regulatory elements seem to be located between  $-159$  and  $-80$  bp, since the highest luciferase activity was obtained with pGL3/159 (Fig. 4C). Extended deletion dramatically reduced promoter activity, with pGL3/80 showing activity not above background. Similar results were obtained with HL60 cells (data not shown). However, with HL60 clone 15 the relative differences in activity between the different deletion-constructions of the promoter were more pronounced. Therefore, all further characterisation was made with HL60 clone 15 cells.

#### AML-1, SP3, and PU.1 are involved in the transcriptional regulation of BPI

On the basis of the expression pattern in HL60 clone 15 (Fig. 4C), putative *cis*-elements were identified by matching the 159 bp upstream of the translation start with TRANSFAC-database. Several potential sites were identified, including a potential TATA-box at position  $-55$  bp (Fig. 5). To determine their functional significance, several sites were subjected to site-directed mutation, followed by transfection into HL60 clone 15 cells. Mutation of the potential Sp1/Sp3-binding site at  $-136$  bp dramatically decreased the promoter activity (Fig. 6). Moreover, the C/EBP-sites at  $-100$  and  $-77$  bp were shown to be important regulatory elements. However, the C/EBP mutations may also affect potential binding sites for PU.1, C-rel, NF $\kappa$ B, MZF-1, and USF, since these sites are partially overlapping with the mutated C/EBP-sites (Fig. 5). Mutation of AML-1-sites at  $-155$  and  $-142$  bp had a more moderate, but nevertheless significant, effect on the promoter activity. Modification of the MZF-1 putative *cis*-element at  $-69$  bp did not influence the promoter activity at any detectable level (Fig. 6). We conclude that mutations of potential binding sites for Sp1/Sp3, C/EBP, PU.1, AML-1 as well as for C-rel, NF $\kappa$ B, and USF decreased promoter activity in myeloid cells.

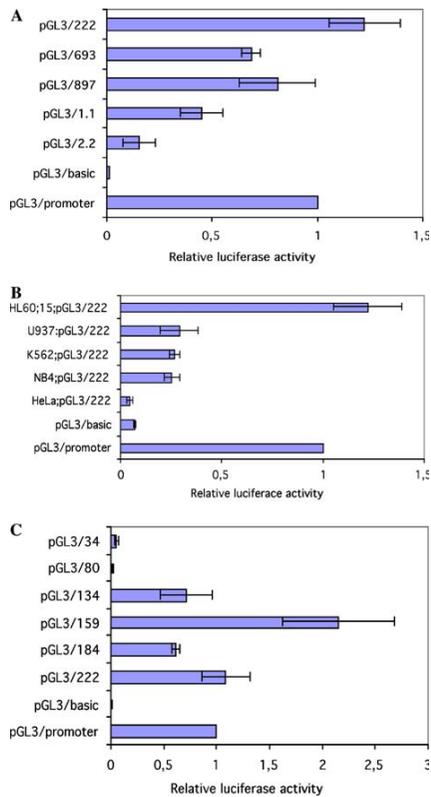


Fig. 4. BPI-promoter activity in myeloid and non-myeloid cells, as shown in a luciferase assay. Progressive 5'-deletion-constructs of the BPI-promoter were transfected to cells, after which luciferase activity was determined. The pGL3/basic and pGL3/promoter are used as a negative and positive control, respectively. The level of luminescence shown is normalised against the luminescence obtained with the pGL3/promoter in each cell line. Absolute levels of luminescence with the pGL3/promoter were: HL60clone15/97 U; U937/243 U; K562/557 U; NB4/4097 U; and HeLa/146 U (mean values). (A) Progressive deletion down to -222 bp results in increasing promoter activity in HL60 clone15 cells. (B) The -222 promoter shows strongest activity in BPI-expressing HL60 clone 15 cells, but also significant activity in myeloid U937, K562, and NB4 cells, while the activity in epithelial HeLa cells is not above background level. (C) Progressive 5'-deletion down to -34 bp defines the region -159/-80 to contain critical elements for promoter activity in HL60 clone 15 cells. The error bars indicate the SEM from at least three different transfection experiments with double samples at each time point.

To identify *trans*-acting proteins binding to the proximal promoter we performed EMSAs using nuclear extract from HL60 clone 15 cells and different oligonu-

cleotide probes corresponding to the promoter. With a probe ranging from -156/-121 bp, including two potential AML-1-binding sites (Figs. 1 and 5), a shift was obtained (Fig. 7). The shift was competed with unlabelled probe or with unlabelled probe containing non-specific mutations (site-directed mutations adjacent to the AML-1-sites). Even a 100-fold molar excess of a probe with the AML-1-binding sites specifically mutated at -155 and -142 could not compete with the shift. Moreover, the shift was supershifted by addition of antibodies against AML-1 (Fig. 7). We conclude that AML-1 binds to the -155 bp and/or -142 site. No supershift was obtained with antibody against Sp1 (Fig. 7). Upon closer examination, however, we observed that the potential Sp1/Sp3-site in the oligonucleotide probe was located close to the end of the probe. We therefore extended the probe to include flanking sequences potentially important for Sp1/Sp3-binding. Indeed, using a -149/-108 bp oligonucleotide probe we could demonstrate a specific shift and supershift, upon incubation with antibodies against Sp3 (Fig. 8). Repeated attempts to obtain a supershift using two different antibodies to Sp1 were unsuccessful (Fig. 8). These results indicate a direct interaction between AML-1 as well as Sp3 and the promoter.

The probe ranging from -107/-76 bp in the promoter contains potential overlapping C/EBP-, PU.1-, USF-, and C-rel-sites (Figs. 1 and 5). When this oligonucleotide probe was mixed with HL60 clone 15 nuclear extract a shift occurred (Fig. 9A). The shift could be competed with unlabelled probe, but not when it was mutated to inactivate the potential C/EBP-site at -100 bp. Furthermore, it was specific for BPI-expressing cells in as much as the shift was obtained with HL60 clone 15 cells, but not with U937, K562, NB4 or HeLa cells (Fig. 9B). This shows specific, direct binding of a transacting factor. However, since the mutation also affects the potential USF- and PU.1-sites, and perhaps even the adjacent C-rel-site, it is difficult to draw conclusions with respect to the identity of the transacting factor. Supershift assays were therefore performed. Incubation with antibodies to PU.1 resulted in a supershift (Fig. 9C) demonstrating PU.1-binding. In an attempt to identify other transcription factors binding to the probe, antibodies directed against the C/EBP family members C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\delta$ , and C/EBP $\epsilon$ , and against the NF $\kappa$ B complex members NF $\kappa$ B p50, NF $\kappa$ B p65, C-rel, and Rel B were tested in supershift assays. None of these resulted in supershifts, neither did antibodies to USF nor the CAAT-box-binding protein (NFYA and A and B subunits) (data not shown).

To further investigate the role of C/EBP family members in BPI transcriptional regulation, we overexpressed C/EBP $\alpha$  and C/EBP $\epsilon$  in HeLa cells. HeLa cells do not express detectable amounts of endogenous BPI (Fig. 2) nor is the proximal BPI-promoter active in these

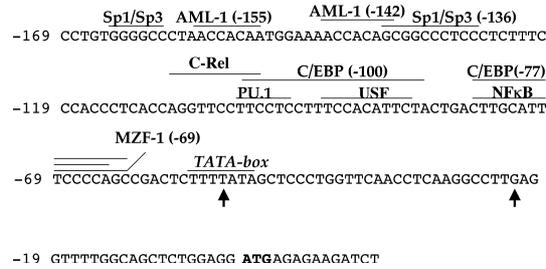


Fig. 5. Sequence of the BPI-promoter and potential transcription factor binding sites. The translation ATG start site is in bold; transcription start sites are indicated with arrows. Potential transcription factor binding sites are indicated.

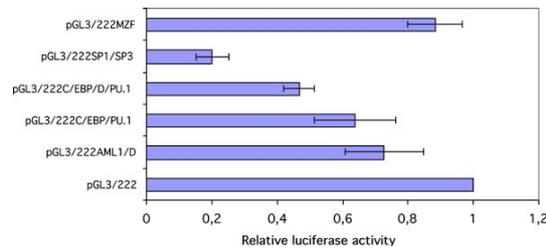


Fig. 6. Effects of mutations of potential regulatory *cis*-elements on BPI-promoter activity. Site-directed mutagenesis of potential MZF1(-69), Sp1/Sp3(-136), C/EBP(-100, -77), and AML-1(-155, -142) sites was performed as described in the Materials and methods, after which transfection to HL60 clone 15 cells was performed. The promoter activity of mutated constructs is normalised against that of non-mutated pGL3/222. In pGL3/222AML1/D two AML1-sites are mutated, in pGL3/222C/EBP and pGL3/222C/EBP/D one (-100) and two sites (-100, -77) are mutated, respectively. The C/EBP(-100) mutation also affects a potential PU.1-site (see text). The error bars represent the SEM from at least three different transfection experiments with double samples at each time point.

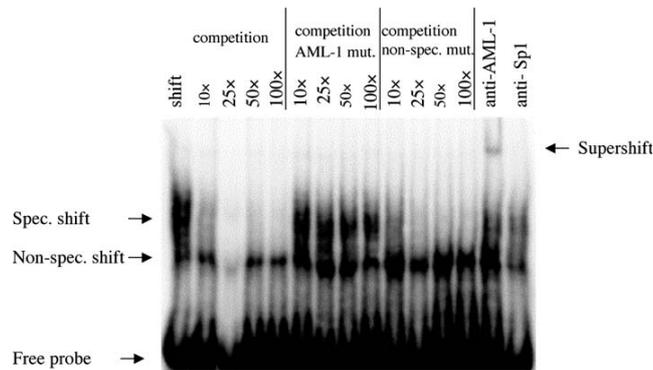


Fig. 7. EMSA indicating direct binding of AML-1 to the BPI-promoter. A probe corresponding to -156/-121 bp, including the potential AML-1-binding sites (Figs. 1 and 5), was used in EMSA with HL60 clone 15 nuclear extract. A specific shift was obtained that was competed by increasing excess of unlabelled probe (competition), but not with unlabelled probe including two mutated AML-1-sites (competition, specific). A non-AML1-site-related mutation retained the capacity for competition (competition, non-specific), further supporting the specificity of the shift. Addition of antibody to AML-1 resulted in a supershift. The specific shifts, non-specific shift, AML-1 supershift, and free probe, are indicated with arrows.

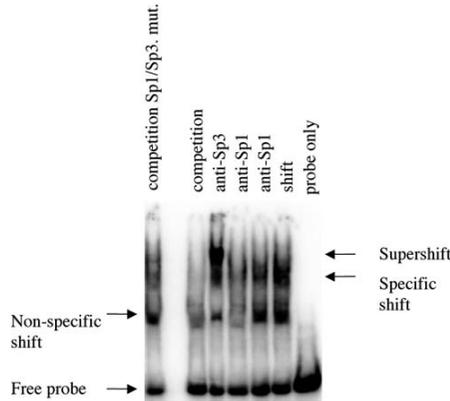


Fig. 8. EMSA indicating direct binding of Sp3 to the BPI-promoter. A probe corresponding to –149/–108 bp, including a potential Sp1/Sp3-site (Figs. 1 and 5), was incubated with HL60 clone 15 nuclear extract. A shift is shown that was competed by addition of a 20× molar excess of unlabelled probe (competition), but not by an identical excess of probe with mutated Sp1/Sp3-site (competition, specific mutation), indicating specificity of the shift. A supershift was observed by addition of antibody to Sp3, indicating the presence of Sp3 in the DNA/protein complex. No shift was observed upon addition of two different antibodies to Sp1. The specific shifts, non-specific shift, Sp3 supershift, and free probe, are indicated with arrows.

cells (Fig. 4B). Overexpression of C/EBP $\alpha$  or C/EBP $\epsilon$  strongly induced transcription from the –222 bp promoter (Fig. 10), demonstrating that C/EBP can, directly

or indirectly, activate the proximal BPI-promoter. These data suggest a role of C/EBP in the activation of the BPI-promoter. Indeed, when assaying expression of endogenous BPI in HeLa cells with RT-PCR, overexpression of C/EBP $\alpha$  or C/EBP $\epsilon$  induced detectable levels of BPI transcript (data not shown), giving further support to the notion that C/EBP is a direct or indirect transactivator of the BPI-promoter. To exclude that upregulation was due to LPS contaminating the vector

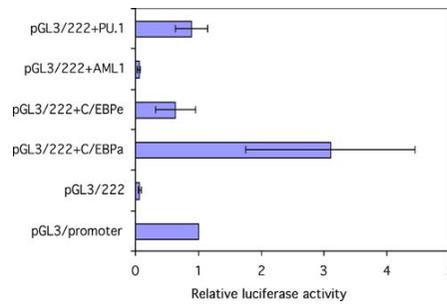


Fig. 10. Overexpression of C/EBP in HeLa cells induces BPI-promoter activity. Expression vectors for C/EBP $\alpha$ , C/EBP $\epsilon$ , AML-1, PU.1 or empty pcDNA3, together with pGL3/222, were transfected to HeLa cells. A strong promoter activity after transfection with C/EBP $\alpha$ , C/EBP $\epsilon$  or PU.1, but not with AML-1, is shown. Luciferase values are normalised to those of pGL3/promoter. Bars: SEM from at least three different transfection experiments with double samples at each time point.

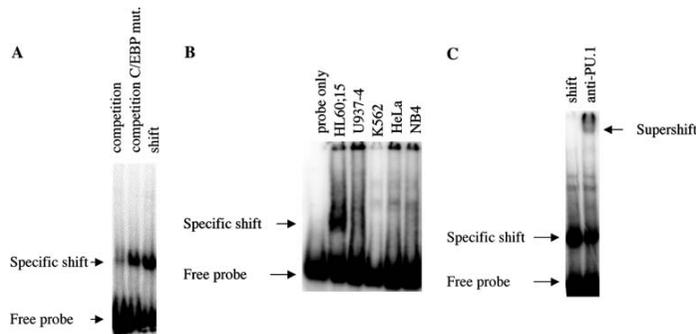


Fig. 9. EMSA indicating direct binding of PU.1 to the BPI-promoter. A probe corresponding to –107/–76 bp, including putative C/EBP-, PU.1-, C-rel-, and USF-binding sites (Figs. 1 and 5), was incubated with nuclear extract from different cell lines. The specific shifts, as well as free probe, are indicated with arrows. (A) Using nuclear extract from HL60 clone 15 cells, a mobility shift is shown that is competed by addition of a 20× molar excess of unlabelled probe (competition), but not by identical excess of probe with mutated C/EBP-site (competition-specific mutation), indicating specificity of the shift. (B) Shifts were not obtained with nuclear extracts from U937, K562, HeLa or NB4 cells, thus indicating a correlation between shift and BPI-expression. (C) The specific shift obtained with HL60 clone 15 nuclear extract was supershifted after addition of antibodies to PU.1. The specific shifts, non-specific shift, PU.1 supershift, and free probe, are indicated with arrows.

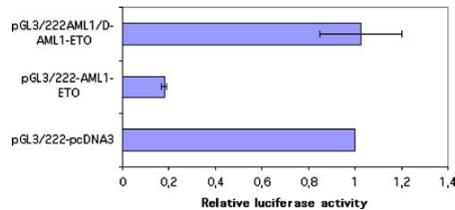


Fig. 11. AML1-ETO represses BPI-promoter activity. PGL3/222 was transfected to HL60 clone 15 cells together with 10  $\mu$ g of expression vectors for AML1-ETO or empty pcDNA3. The experiment was repeated with pGL3/222AML1/D, with two AML1-sites mutated at -155 and -142 bp. AML1-ETO strongly reduces BPI-expression in an AML1-site-dependent manner. Luciferase values are normalised to those of control (pcDNA3). Bars: SEM from at least three different transfection experiments with double samples at each time point.

preparation, HeLa cells were induced with LPS. However, no induction of BPI-expression was detected (data not shown). In the same experimental setting, overexpression of PU.1 did also result in a similar promoter activation (Fig. 10), further corroborating the role of PU.1 as a direct activator of BPI-expression.

Upon overexpression of AML1-1b in HeLa cells, however, no activation was obtained (Fig. 10). Given that mutation analysis and EMSA indicated an important role of AML1, this finding was unexpected and might indicate that AML1 requires additional factors, such as CBF $\beta$  [32–35] or MOZ [36], to be active. The fusion protein AML1-ETO, present in some cases of acute myeloid leukemia, functions as a strong repressor of AML1-responsive promoters in a dominant negative fashion [37–40]. To gain further support for participation of AML1 in BPI-expression, we therefore overexpressed AML1-ETO and determined its effect on promoter activity. Indeed, when AML1-ETO was overexpressed in HL60 cells, BPI-promoter activity was strongly reduced (Fig. 11). Moreover, the inhibitory effect of AML1-ETO was dependent on binding to AML1 *cis*-elements, since no reduction was seen when AML1-binding sites were mutated (Fig. 11). These results corroborate the fact that AML1 indeed binds to the BPI-promoter *in vivo*.

## Discussion

In this work the proximal promoter of human BPI is structurally and functionally characterised. Our data define a proximal promoter with a myeloid-specific transcriptional activity contained within the 222 bp upstream of the translation start. The promoter is directly activated by the transcription factors AML-1, PU.1, and Sp3 and directly or indirectly by members of the C/EBP-family.

A myeloid specificity of the proximal promoter of BPI was demonstrated by activity after transfection to myeloid HL60, U937, K562, and NB4 cells, but not to non-myeloid HeLa-cells. Furthermore, a correlation between promoter activity and expression of the endogenous BPI gene was found, since HL60 and HL60 clone 15 cells (both expressing BPI) showed by far the strongest activity, as judged by activity compared to the SV40-promoter of pGL3/promoter (Fig. 4B). Thus, the promoter is weakly activated by factors generally present in myeloid cells, but is strongly active in cells expressing the BPI gene.

Which factors are then critical for expression of the BPI gene? Since maximal promoter activity was retained in the 159 bp region and since the activity of this promoter showed a strong correlation to endogenous BPI-expression, it seems reasonable to suggest that *cis*-regulatory elements important for gene expression should be present here. Among the potential *cis*-elements found were AML-1, Sp1/Sp3, C/EBP, USF, MZF-1, PU.1, C-rel, and NF $\kappa$ B included (Fig. 5). Our findings that site-directed mutations of AML-1 or Sp1/Sp3 *cis*-element resulted in a moderate or dramatic reduction, respectively, of the promoter activity (Fig. 6) indicate that these transcription factors are important for promoter activity. This hypothesis was further supported by the demonstration that AML1 and Sp3 bind directly to the promoter (Figs. 7 and 8).

AML-1 has previously been shown to be involved in the regulation of other azurophil granule proteins, such as myeloperoxidase (MPO) and elastase [41]. To our knowledge, no specific granule protein has been reported to be regulated by AML-1. Two AML-1 *cis*-elements present in the promoter are located adjacent to *cis*-elements for Sp1 and Sp3 (Fig. 5). Sp1 and Sp3 are ubiquitously expressed transcription factors (reviewed in [42]). Sp1 is involved in the regulation of both azurophil granule proteins, e.g., elastase [43] and specific granule proteins e.g., lactoferrin, CD18 [44,45]. Sp3 can act either as a transcriptional activator or as a repressor, depending on the promoter and cellular context [46] and is essential for erythroid and myeloid haematopoiesis [47]. Since mutation of the Sp1/Sp3-site dramatically interfered with promoter activity (Fig. 6), and since Sp3 was shown to directly bind to the promoter (Fig. 8), we conclude that Sp3 functions as an activator of the BPI-gene in HL60 cells. It cannot, however, be excluded that Sp3 can act as a repressor of BPI in other myeloid cells. The BPI-promoter characterised here and the lactoferrin promoter [44] have a similar organisation with respect to the Sp1/Sp3-sites, two flanking Sp1/Sp3-sites surrounding tissue-specific factor binding sites, AML-1 in the BPI-promoter and C/EBP in the case of the lactoferrin-promoter. In both cases these regulatory clusters are located in close proximity, upstream of the transcription start. To our knowledge, however, adjacent functional

AML-1 and Sp1/Sp3-sites have not been previously described in myeloid promoters. Since AML-1b alone was incapable of activating the transcription of BPI after transient transfection to HeLa-cells (Fig. 10) that express high levels of Sp1 and Sp3 [42], it is likely that the activity of AML-1b depends on cooperation with additional transcription factors. However, no additive effects were seen upon activation, using different combinations of AML-1 and C/EBP $\alpha$  or C/EBP $\epsilon$  (data not shown). The lack of effect of AML-1b might be explained by the fact that AML-1b often requires partners like CBF $\beta$  [32–35] or MOZ [36] to transactivate a promoter. These factors may be absent in HeLa cells. However, our finding that AML1-ETO almost completely abolished BPI-promoter activity when overexpressed in HL60 clone 15 (Fig. 11) strongly indicates that AML1 regulates the BPI-promoter in vivo. The ETO moiety in AML1-ETO has previously been shown to recruit with histone acetyl deacetylases and thereby to repress transcription [37–40]. Since the repressor effect of AML1-ETO was dependent on functional AML-1-sites (Fig. 11), the effect was specific. The specific AML1-ETO-binding to AML1 *cis*-elements and resulting repression of the promoter emphasise that AML-1 indeed binds and transactivates BPI-promoter in vivo.

Overexpression of Sp1 and Sp3 in HeLa cells was not performed, since HeLa cells express high levels of these factors [42]. Sp1 is often involved in the regulation of promoters that lack a TATA-box, [48] a characteristic sign of several promoters for neutrophil granule proteins (reviewed in [49]). It is unclear whether the promoter for BPI contains a functional TATA-box. A putative TATA-box is present in the core promoter at –55 bp, but since the location overlaps with the transcription start at –52 bp, its functional significance is questionable. However, the location of the alternative transcription start at –22 bp is consistent with a functional TATA-box at –55 bp.

PU.1 is a key myeloid transcription factor [50] that is involved in the transcriptional regulation of several azurophil and specific granule proteins, reviewed in [49,51]. PU.1 was found to participate in the regulation of BPI via direct binding to its promoter (Fig. 9C). The importance of PU.1 was further highlighted by its capacity to activate pGL3/222 when transfected into HeLa cells (Fig. 10). Moreover, BPI-promoter activity was decreased when the PU.1-binding site was modulated (Fig. 6). PU.1 and AML1 have been shown to synergistically cooperate via their transactivating domains [52]. A similar scenario is likely to occur in the BPI-promoter, even though further investigation is needed to demonstrate this. However, it was unexpected that no shift was detected in the other myeloid cell lines investigated when using the PU.1-binding oligonucleotide probe (Fig. 9B), since some of these cell lines express PU.1 [53]. Interestingly, when acute promyelocytic leu-

kaemia cells are treated with ATRA, PU.1 binds to a higher degree to the M-CSF receptor promoter [54]. Therefore, unidentified cooperating factors may enhance PU.1-binding to the BPI-promoter in HL60 cells.

C/EBP-family members often participate in the transcriptional regulation of genes coding for azurophil and specific granule proteins (reviewed in [55,56]). Two potential C/EBP-sites were found in the proximal BPI-promoter (Fig. 5). Mutation of one or both of these C/EBP-sites progressively decreased the promoter activity (Fig. 6). However, the –100 bp site overlaps with a PU.1-site, which may also be affected by the mutation. Therefore, both C/EBP and PU.1 potentially bind. Indeed, our findings that the PU.1-antibody induced a supershift (Fig. 9C) demonstrate that the potential PU.1-site (overlapping with the C/EBP-site) is functional in HL60 clone 15 cells. However, a role also for C/EBP in BPI regulation is suggested by our findings that overexpression of C/EBP $\alpha$  and C/EBP $\epsilon$  in HeLa cells dramatically activates the BPI-promoter activity (Fig. 10).

In conclusion, we have defined the core promoter of BPI and demonstrate a 222 bp proximal promoter that confers a myeloid-specific expression. Moreover, we have identified a 159 bp minimal promoter region as critical for the activity in myeloid cells. Within this region AML-1, Sp3, and PU.1 bind directly and activate expression. AML-1 and PU.1 participate in the regulation of several myeloid genes, but this is the first report of Sp3 in the transactivation of a gene encoding a neutrophil azurophil granule protein.

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