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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/EBPα or C/EBPε 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.

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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 55kDa 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 α (TNFα) 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].
BPI is stored together with a wide range of other bac-
tericidal proteins and antimicrobial polypeptides, e.g.,
serine proteases and defensins in the azurophil granules.
Formation of neutrophil granules occurs sequentially
during maturation; azurophil 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 synthe-
sised prior to those of specific granules. However, the ex-
pression of different azurophil proteins is not completely
synchronized, 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 az-
urophil granules in the newborn are specifically deficient
in BPI, whilst still being equipped with normal amounts of
other azurophil granule proteins such as myeloperoxidase
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 homo-
ygous elimination of the neutrophil transcription factor
C/EBPb [22]. Elimination of C/EBPb in mice resulted in a
phenotype resembling human SGD [23]. However, since
murine neutrophils do not contain BPI [24] it is not pos-
tible to determine the effects on BPI in this animal model.
Thus, previous observations indicate that the tran-
scriptional 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
expression 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 BPI, whilst still being equipped with normal amounts of
other azurophil proteins such as myeloperoxidase.
Material and methods

Cell culture. U937-4, a subclone of U937 [25] HL60, K562, and
HL60 cells were maintained in RPMI-1640 medium (GIBCO) sup-
plemented with 10% fetal calf serum (FCS, GIBCO). HL60 clone 15
(CTEC 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 μg total RNA was performed using the GeneAmp RNA PCR-kit
(Perkin Elmer). The primer 5′-TGAGAGTGCCAGCAGGACA
TAGGT-3′ was used for reverse transcription and also in the
subsequent 35 cycle PCR, together with the upstream primer
5′-ATGAGAAGAACAGATGCCAGGCCG-3′.

Northern blot. Northern blot was performed with the Espra-
Hybridisation solution (Clontech). Fifteen μg total RNA was sepa-
rated on a formaldehyde gel followed by transfer to a Genome x
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 [32P]dCTP using the
Rediprime II kit (Amersham–Pharmacia Biotech). High stringency
hybridisation was performed at 68 °C. The membrane was washed with
2× SSC, 0.1% SDS at 37 °C for 30 min; and 0.1 SSC, 0.1% SDS 2× 20 min at
50 °C. Membranes were analysed using a Molecular Imaging FX analyzer (BioRad).

Genomic cloning. Cloning of genomic DNA upstream of the tran-
scription start was performed by PCR utilizing the 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′-TGACGCGCTGCTTCTATCG
CGCA-3′ and nested primer 5′-TCGAGATCTTCTCTATCTCC
GAACGTCCAAAAACCTC-5′ (RIPHI 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 (trans-
scription start) was identified by 5′-rapid amplification of cDNA-ends
(RACE) using the 5′RACE kit (Roche) or the First Choice RLM-race
kit (Ambion). Nested PCR in the RACE reactions was performed with
adapter primer 5′-GACGCGCTGCTTCTATCGCGCA-3′ and nested primer
5′-GACGCGCTGCTTCTATCGCGCA-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 promoters 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. PCR amplification according to the transcription 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 cre-
ated with the Enzyma-base System (Promega). For site-directed mu-
tagenesis of potential transcription factor binding sites, oligonucleotide
primers including the desired mutations were synthesized and used in two-step splice overlap extension (SOE) PCR as described [27].
The following potential transcription binding sites were mutated: CEBP-
site at position −186 bp, 5′-CTTCG-3′ changed to 5′-GGGGA-3′,
CEBP-site at position −77, 5′-CATT-3′ changed to 5′-AAGG-3′;
AML-1-site at −142 bp, 5′-ACCAC-3′ changed to 5′-CAACAC-3′;
AML-1-site at −142 bp, 5′-ACCAC-3′ changed to 5′-CAACAC-3′;
MZF-1-site at −316 bp, 5′-CGTCT-3′ changed to 5′-TAAGG-3′;
MZF-1-site at −669 bp, 5′-CCCGA-3′ changed to 5′-AACAC-3′. In all
constructs only one binding site was mutated, with the exception of
pGL3/222AMLL1D in which both sites (−155 and −342) were modified and pGL3/222C
(both C/EBP-sites at −100 and −77) were mutated. After
subcloning into pGL3/basic, mutations were verified by sequencing.

Reverse transcription PCR. Reverse transcription PCR (RT-PCR)
of 1 μg total RNA was performed using the GeneAmp RNA PCR-kit
(Perkin Elmer). The primer 5′-TGAGAGTGCCAGCAGGACA
TAGGT-3′ was used for reverse transcription and also in the
subsequent 35 cycle PCR, together with the upstream primer
5′-ATGAGAAGAACAGATGCCAGGCCG-3′.
medium. Electroporation was performed in a 0.4 cm cuvette (Gene-pulser II, BioRad) at a capacitance of 960 μF. 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 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 μg DNA/340 V/5 × 10⁶ cells/16 h; HeLa, 15 μg DNA/800 V/5 × 10⁶ cells/16 h; U937, 15 μg DNA/280 V/5 × 10⁶ cells/16 h; and K562, 15 μg DNA/280 V/6 × 10⁶ 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/EBPα, C/EBPβ, PU.1, AML1-ETO, and AML-1b, expression vectors were used (vectors were kindly provided by Dr. Pierre A. Iman, Gothenburg University, Sweden, Dr. Phil Koehler, UCLA, Los Angeles, USA, Dr. Mikael Sigvardsson, Lund University, Lund, Sweden, and Dr. Scott Hubert, St. Jude Children’s Research Hospital, Memphis, USA, respectively). HeLa cells were transfected with Lipofectin reagent (Invitrogen Life technologies).

Expression of the BPI gene in haematopoietic cells

Results

Expression of the BPI gene in haematopoietic cells

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

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.
the CDNA sequence was identical in both PCR products. Further sequencing was therefore confined to the 2.2kb 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 1.1kb promoter sequence to the transcription factor database TRANSFAC [29] with various matches, the 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 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-constructs 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 159bp upstream of the translation start with TRANSFAC Database. Several potential sites were identified, including a potential TATA-box at position −55bp (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 −136bp dramatically decreased the promoter activity (Fig. 6). Moreover, the C/EBP-sites at −100 and −77bp were shown to be important regulatory elements. However, the C/EBP mutations may also affect potential binding sites for PU.1, C-rel, NF-kB, 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 −142bp had a more moderate, but nevertheless significant, effect on the promoter activity. Modification of the MZF-1 putative cis-element at −69bp 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, C-rel, NF-kB, and USF decreased promoter activity in myeloid cells.
To identify trans-acting proteins binding to the proximal promoter we performed EMSAs using nuclear extract from HL60 clone 15 cells and different oligonucleotide 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/EBPα, C/EBPβ, C/EBPδ, and C/EBPε, and against the NFκB complex members NFκB p50, NFκB 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/EBPs 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. 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 AML1(-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 AML1-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 AML1-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 AML1 resulted in a supershift. The specific shifts, non-specific shift, AML1 supershift, and free probe, are indicated with arrows.
cells (Fig. 4B). Overexpression of C/EBPα or C/EBPε 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/EBPα or C/EBPε 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. 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.

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. 10. Overexpression of C/EBP in HeLa cells induces BPI-promoter activity. Expression vectors for C/EBPα, C/EBPε, AML-1, PU.1 or empty pcDNA3, together with pGL3/222, were transfected to HeLa cells. A strong promoter activity after transfection with C/EBPα, C/EBPε or PU.1, but not with AML-1, is shown. Luciferase values are normalised to those of pGL3promoter. Bars: SEM from at least three different transfection experiments with double samples at each time point.
AML1 indeed binds to the BPI-promoter in vivo. These results corroborate the fact that no reduction was seen when AML1-binding sites were mutated (Fig. 11). Moreover, the inhibitory effect of AML1-ETO was strongly reduced in HL60 cells, BPI-promoter activity was strongly reduced upon overexpression of AML-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 or MOZ, 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. To gain further support for participation of AML1 in BPI-expression, we therefore overexpressed AML1-ETO and determined its effect on promoter activity. This hypothesis was further supported by the demonstration that AML1 and Sp3 bind directly to the promoter (Figs. 7 and 8).

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. 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, MZF1, PU.1, C-rel, and NFkB 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).

AML1 has previously been shown to be involved in the regulation of other azurophil granule proteins, such as myeloperoxidase (MPO) and elastase. To our knowledge, no specific granule protein has been reported to be regulated by AML-1. Two AML1 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 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, AML1 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. 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/EBPα or C/EBPγ (data not shown). The lack of effect of AML-1b might be explained by the fact that AML-1b often requires partners like CBFα [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 AML1-sites (Fig. 11), the effect was specific. The specific AML1-ETO-binding to AML1 cis-elements and resulting repression of the promoter emphasise that AML1 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 leukaemia 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/EBPα and C/EBPγ 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 AML1, Sp3, and PU.1 bind directly and activate expression. AML1 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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