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

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

Doctoral Thesis

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Division of Hematology and Transfusion Medicine
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Kunskap

Vad gjorde oss mänskliga?
Inte alltid kärleken.
Mycket sällan njutningen.
Kanske oftare lidandet.
Måhända intelligensen?
Jo, allra mänskligast
var vår iver att avnjuta
intellektets smak för
negationens bittra sötma.
Förkasta för en upptäckt.
Åh, den där ambitionen
att skapa en ny värld –
som om vi känt en gammal.
Så kom vi då fram en bit
på vägen, ängsliga, gamla,
men likt yrvakna skolbarn,
välkammade, vi bar namn-
brickor, små tygväskor,
i dem förvarade vi våra
hemliga anteckningar
om livets mening.

Jacques Werup

Ur Hundra Dikter från fyra decennier.

Abstract

In myelopoiesis, neutrophils and monocytes/macrophages are formed from a common myeloid precursor cell in the bone marow. During maturation of neutrophils, cytoplasmic granules are formed in the cells and the content of these granules is critical for the function of neutrophils in the first line of defense against invading microorganisms. The Bactericidal/Permeability-Increasing protein (BPI) is stored in azurophil granules of the neutrophil and is cytotoxic against gram-negative bacteria. Absence of BPI in neutrophils of patients with Specific Granule Deficiency (SGD) and late expression of BPI at the promyelocyte stage, suggest a different regulation at the transcriptional level as compared to other azurophil granule proteins. In this thesis, we show that the transcription factors AML-1, PU.1, and Sp3 regulate the expression of human BPI in myeloid cells. We also identified a mouse ortholog to human BPI, expressed in bone marrow, testis and epididymis. However, the expression of BPI in mouse bone marrow cells is much lower than that in human bone marrow cells, indicating important differences in the transcriptional regulation. We suggest that, in contrast to human BPI, lack of C/EBP binding sites is responsible for the low expression of BPI in mouse bone marrow cells.

The expression of another azurophil granule protein, human proteinase 3, is shown to be increased in myeloid cells of patients with Wegener's Granulomatosis (WG). This increased expression of proteinase 3 might be a risk factor for the development of the disease. We show that the overrepresented –564 A/G SNP in WG patients, which introduces a new potential Sp1 transcription factor binding site, is not the reason for increased proteinase 3 expression in these patients.

Original papers

This thesis is based on the following papers, which are referred to in the text by their respective roman numerals.

I. Lennartsson A., Pieters K., Ullmark T., Vidovic K, and Gullberg U. (2003) AML-1, PU.1, and Sp3 regulate expression of human bactericidal/permeability-increasing protein. Biochemical and Biophysical Research Communications, 311: 853-863.

II. Lennartsson A., Pieters K., Vidovic K., and Gullberg U. (2005) A murine antibacterial ortholog to human bactericidal/permeability-increasing protein (BPI) is expressed in testis, epididymis, and bone marrow. Journal of Leukocyte Biology, 77: 369-377.

III. Pieters K, Lennartsson A, Gullberg U. Cloning and characterization of the mouse bactericidal/permeability-increasing protein (BPI) gene promoter. Manuscript.

IV. Pieters K., Pettersson Å., Gullberg U., and Hellmark T. (2004) The –564 A/G polymorphism in the promoter region of the proteinase 3 gene associated with Wegener's granulomatosis does not increase the promoter activity. Clinical and Experimental Immunology, 138: 266-270.

Contents

Selected abbreviations

AASV	ANCA-associated small vessel vasculitis
AML	acute myeloid leukemia
ANCA	anti-neutrophil cytoplasmic autoantibodies
APL	acute promyelocytic leukemia
ATRA	all-*trans*-retinoic acid
BPI	bactericidal/permeability-increasing protein
C/EBP	CCAAT/enhancer binding protein
CD	cluster of differentiation
CDP	CCAAT displacement protein
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-monocyte-colony stimulating factor
GTF	general transcription factor
HAT	histone acetyl transferase
HDAC	histone deacetylase
IL	interleukin
LBP	LPS-binding protein
LPS	lipopolysaccharide
M-CSF	monocyte-colony stimulating factor
MPO	myeloperoxidase
NE	neutrophil elastase
PLUNC	palate, lung and nasal epithelial clone
PMN	polymorphonuclear neutrophil
PR3	proteinase 3
RARα	retinoic acid receptor-alpha
RARE	retinoic acid response element
RTF	regulatory transcription factor
SGD	specific granule deficiency
TBP	TATA-binding protein
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
WG	wegener's granulomatosis

I. Background

A. Haematopoiesis, general features

Haematopoiesis means formation of blood cells and is a process which occurs in the bone marrow. A rare population (<0,1%) of pluripotent stem cells have the ability to self-renew as well as to differentiate to committed multipotential haematopoietic precursor cells. The human haematopoietic system has an enormous amplification rate: one stem cell is capable of producing about 1 million mature blood cells after 20 cell divisions and this production continues during human lifetime.

The committed multipotential precursor cells are highly proliverative cells that express receptors for specific growth-and survival factors, like colony-stimulating factors (CSF's), and after differentiation finally form seven distinct classes of mature blood cells, including erythrocytes, B cells, T cells, NK cells, granulocytes, monocytes/macrophages, and megakaryocytes. When released from the bone marrow into the blood circulation, each of these mature cell types possess unique properties and perform specific distinct functions. Erythrocytes or red cells function during 120 days in the blood circulation where they transport oxygen (O_2) from the lungs to the tissues where it is exchanged for carbon dioxide (CO_2). The platelets, formed from megakaryocytes, make mechanical plugs during the normal haemostatic response to vascular injury. B cells, T cells, NK cells, granulocytes, and monocytes /macrophages belong to a group of leukocytes which are central to all immune responses in our body. Neutrophils, which together with the eosinophils and basophils, belong to the class of granulocytes, are the most important cells in the first line of defense against invading microorganisms in our tissues, a defense system also called innate immunity. Monocytes/macrophages and neutrophils perform phagocytic functions, which means that they during infection or inflammation migrate into infected or inflamed tissues, bind to the microorganisms, internalize them and then destroy them. Other leukocytes, like B cells and T cells are responsible for the adaptive immune responses and fall into another category of cells, the so called lymphocytes. When B cells have recognized its specific antigen, they divide

and differentiate into plasma cells which produce antibodies specific against the recognized antigens. These plasma cells are memory cells that confer a long-term immunity against the recognized antigens. A second group of lymphocytes are the T cells, which in their turn are subdivided in different types of T cells. T cells express T-cell Receptors (TCR) on their surface for the recognition of specific antigens presented by MHC molecules on the surface of other cells, the so called antigen-presenting cells (APC). One type of T cell is called T-helper cell and can interact with B cells and helps them to divide, differentiate and to produce antibodies. T-helper cells can also interact with macrophages helping them with destroying the ingested pathogens. T-cytotoxic cells are a second type of T cells and interact with host cells infected with virus and other microorganisms and finally destroy these cells. Natural Killer (NK) cells, which are a type of large granular lymphocyte, can destroy infected host cells which lack of have lost their MHC molecules and in this way belongs to the innate immunity [1-3].

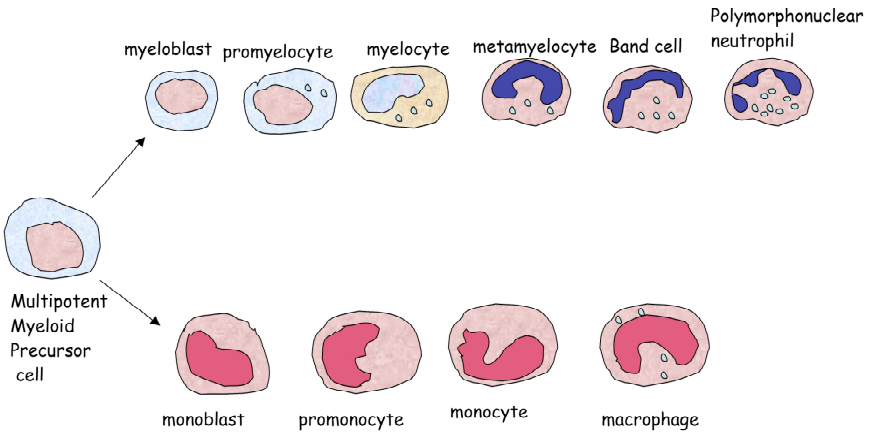

Figure 1. *The differentiation towards neutrophils and monocytes/macrophages is called myelopoiesis.*

B. Myelopoiesis

Neutrophils and monocytes/macrophages all derive from a common myeloid precursor cell and their differentiation process is called myelopoiesis (as illustrated in **figure 1**). During myelopoiesis, cytoplasmic granules of the neutrophil are formed. Cytoplasmic granules are characteristic for these cells and the content of the granules is necessary for the cells to be functionally active. In humans, differentiation of the myeloid cells takes place in the bone marrow over a period of 10 to 14 days, followed by the release of the mature myeloid cells into the peripheral blood [4].

The mature blood neutrophil, also called polymorphonuclear neutrophil (because of its segmented nucleus), contains many types of granules and these are formed at different stages during the myelopoiesis, including the myeloblast stage, promyelocyte, myelocyte, metamyelocyte, band cell, and finally the mature polymorphonuclear neutrophil.

The earliest appearing granules are formed during the promyelocyte stage and are named azurophil (primary) granules followed in time by the specific (secondary), gelatinase (tertiary) granules, and finally the secretory vesicles [5]. The classical distinction between the early-appearing granules (primary) and the later-appearing granules (secondary, tertiary) is based on the fact that primary granules contain the protein myeloperoxidase (MPO), while the other granules not [6]. Further investigations of the structure and protein content of the granules revealed two heterogeneous populations of granules rather than two well-defined granule types [7-10]. The azurophil (primary) granules are further divided in several subpopulations based on heterogeneity in size, shape and protein content [4, 7, 10], but also on differences in time of appearance during the early stage of myelopoiesis. Based on this, azurophil granules can be divided in two major subgroups: early-appearing defensin-poor granules, which are formed at the beginning of the promyelocyte stage, and late-appearing defensin-rich granules, formed at the transition of promyelocyte and myelocyte stage [11]. Alpha-defensins form together with myeloperoxidase (MPO) major consituents in azurophil granules, but also proteins like bactericidal/permeability-increasing protein (BPI) and the closely related serine protease homologues neutrophil elastase (NE), proteinase 3 (or myeloblastin),

azurocidin, and cathepsin G are found in these granules. The secondary (specific) and tertiary granules, which do not contain myeloperoxidase (MPO), can be divided into three subgroups based on the content of lactoferrin and gelatinase: 15% of these granules contain lactoferrin but no gelatinase; 60% contain lactoferrin and gelatinase and 25% contain gelatinase but no lactoferrin [9]. The expression of these peroxidase-negative granules occcurs as a continuum in myelocytes, metamyelocytes, band cells and segmented neutrophils and besides lactoferrin and gelatinase, they also contain the peptide hCAP-18, lipocalin, lysozyme (which is found in all granule types) and collagenase.

This granule heterogeneity separates proteins that cannot co-exist in the same granule [12] and is explained by differences in timing of gene expression. Arnljots and co-workers found that major differences exist in timing of biosynthesis of MPO, defensins and lactoferrin in relation to maturation of myeloid cells and that this can explain the differences in subcellular localization of the individual granule proteins [11].

The gene expression of the individual granule proteins during myelopoiesis is regulated at different levels and one of the main regulatory mechanisms is at the level of access to the DNA sequences, that is, at the chromatin level. Genes or DNA sequences are very tightly assembled into chromatin, which make them transcriptionally inactive. The conformational changes of chromatin, also called remodeling of chromatin, makes genes accessible to bind DNA-binding proteins (transcription factors). The initiation or start of transcription is 'primary' regulated by the interplay of molecular interactions between transcription factors and promoter and/or enhancer sequences. At this level of transcription, DNA is converted into primary RNA transcript which undergoes post-transcriptional modifications to form a mature mRNA (messenger RNA). This mRNA is then transported from the nucleus to the cytoplasm, where it is translated into protein which is subjected to different types of post-translational events. Lots of studies have been done on post-translational mechanisms, like processing and targeting of the granule proteins in neutrophils [13], as well as studies on regulation of gene expression at the transcriptional level [14, 15].

In this thesis, I have focused on the transcriptional regulation of the gene expression of Bactericidal/Permeability-Increasing protein (BPI) and proteinase 3. Human BPI and proteinase 3 are stored in the azurophil granules of the neutrophil granulocyte, but human BPI is also found in some epithelial tissues. We also cloned and identified a mouse ortholog to human BPI which was found to be expressed in bone marrow cells, but also in testis-and epididymis tissue. A study of the promoter structure of this 'novel' mouse BPI was initiated and some interesting, although preliminary, differences between the transcriptional regulation of human and mouse BPI were found. All three proteins, human BPI, human proteinase 3, and probably also mouse BPI play specific roles in innate immunity and this will be described in more detail later in this thesis.

B. 1. The role of transcription factors in myelopoiesis

Lots of studies have been made to understand how myeloid precursor cells undergo commitment and become mature neutrophil granulocytes or monocytes/macrophages. The action of transcription factors in response to specific growth factors or interleukins play a major role in both commitment and maturation of myeloid cells. Transcription factors can act both positively and negatively to regulate the expression of genes encoding for typical granule proteins, but also expression of specific growth factors and their receptors and expression of other transcription factors.

B. 1. 1. Transcription factors and lineage choice

PU.1 is one of the crucial transcription factors identified to regulate the differentiation of myeloid cells. PU.1 seems to be required for the development of the early multipotential myeloid precursor cells [16, 17] and plays also an essential role in the development of monocytes/macrophages later during myelopoiesis [18, 19]. The precise mechanisms of the differentiation from the committed multipotential myeloid precursor cell into either neutrophils or monocytes/macrophages is still not fully understood. One hypothesis [20] is that the continued expression of PU.1 leads to the development of the 'default'

pathway, this means the differentiation of the myeloid progenitor cells into monocytes/macrophages. According to this model (see schematic representation in **figure 2**), C/EBPα, another important transcription factor during myelopoiesis, can inhibit the function of PU.1 and up-regulation of the activity of C/EBPα results in induction of the granulocytic development and inhibition of monocytic development [21, 22].

PU.1 regulates almost all the myeloid genes including the receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) and granulocyte colony-stimulating factor (G-CSF) [19, 23-25]. The differentiation into monocytes/macrophages is dependent on the expression of the receptor for M-CSF and the monocyte/macrophage differentiation process can only take place efficiently if the cells respond to this specific growth factor [18]. PU.1 deficient cells can commit into the granulocytic and monocytic lineages, but cannot fully support maturing along these lineages [26-28]. C/EBPα deficient mice fail to undergo myeloid differentiation beyond the myeloblast stage and therefore lack mature neutrophils, but show no lack of mature monocytes [29]. In contrast to PU.1, loss of C/EBPα affects the expression of the G-CSF receptor, but not the receptors for GM-CSF or M-CSF [25].

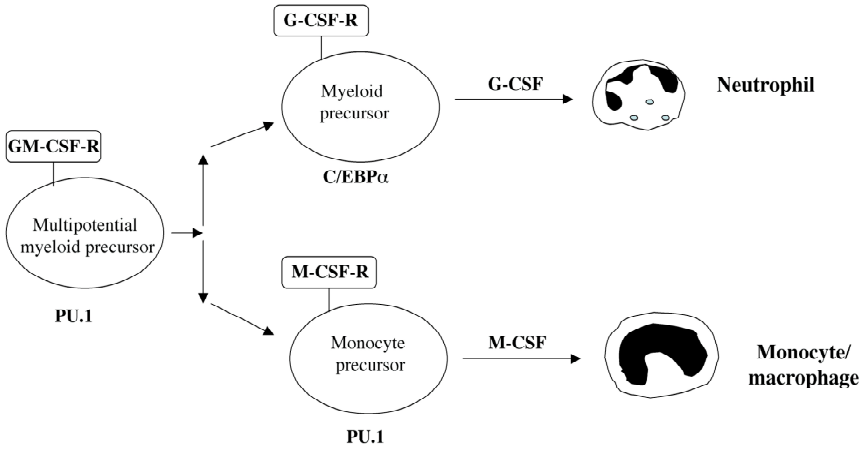

Figure 2. *Schematic representation of myeloid lineage choice. Modified from Tenen, 1997.*

B. 1. 2. Transcription factors and receptor-mediated signals

The activity of several transcription factors during myelopoiesis is controlled by external stimuli, such as cytokines and specific growth factors. Activation of transcription factors, such as PU.1 and C/EBPα, induces expression of specific cytokine receptors (**Figure 2**). One of these, the granulocyte colony stimulating factor (G-CSF) receptor regulates the production of granulocytes [30]. Signals from this G-CSF receptor activate c-Jun, the co-activator of PU.1, and both proteins bind to the promoters of several myeloid genes resulting in the induction of differentiation of myeloid cells [31, 32].

According to one model, transcription factors rather than cytokine receptor signals are key regulators in myelopoiesis [33]. This hypothesis is based on some data in the literature including the observation that mice lacking G-CSF, the G-CSF receptor, or G-CSF and GM-CSF, have a reduction in granulocyte production, indicating their importance for myelopoiesis [30]. However when compared to mice lacking the transcription factors PU.1 or C/EBPα, the impairment in granulocyte production was much greater in the latter case [17, 29, 34], demonstrating the critical importance of transcription factors. Furthermore, the introduction of the G-CSF receptor into PU.1 deficient bone marrow cells failed to restore terminal myeloid differentiation [35].

Myeloid differentiation can also be induced by the Retinoic Acid Receptor-alpha (RARα) which function both as a transcription factor and a nuclear receptor able to bind retinoic acid (RA), a small lipophilic molecule that is derived from retinol, or vitamin A [36-38]. A dominant-inhibition of RARα arrests myelocytic differentiation at the promyelocyte stage [39], while treatment of the leukemic NB4 cells by all-trans retinoic acid (ATRA) induced the granulocytic differentiation of these cells and the induction of C/EBPε transcript, which is a direct target gene of RARα [40-43].

B. 1. 3. Transcription factors and disease

The hypothesis [20, 33], as mentioned above, that transcription factors, and not the external growth factors and cytokines are the critical decision points for myeloid differentiation is supported by the fact that many genes which are

involved in chromosomal translocations or mutations in acute myeloid leukemia (AML) are transcription factors [20, 33].

Acute myeloid leukemia (AML) is a disease which is characterized by accumulation of immature myeloid cells blocked in their differentiation program.

The transcription factor AML-1 (or CBFα2), which acts earlier than PU.1 during haematopoiesis, is found to be essential for the expression of some myeloid-specific genes, including M-CSF, GM-CSF, and myeloperoxidase (MPO) [19, 44, 45]. The AML1 gene is located at the translocation breakpoint on chromosome 21 in the t(8; 21) translocation and forms the fusion gene AML1/ETO, which encodes the most common fusion protein found in AML patients [46]. The fusion protein consists of the N-terminal part of AML1 fused to almost the complete ETO protein [47] and represses genes that are normally activated by wild type AML1 [44]. Further, the AML1/ETO fusion protein disrupts C/EBPα function [48] and might also inhibit PU.1 function [49]. Some AML patients which do not have this common translocation t(8; 21) are found to have mutations either in the PU.1 gene [50] or in the C/EBPα gene [51]. The mutations in the C/EBPα gene are divided into two common types: mutations in the C-terminus which disrupts the DNA binding as well as homo- and heterodimerization with other C/EBP family members, and N-terminal frameshift mutations resulting in a truncated protein [51]. The truncated protein is also inhibited in its DNA-binding ability [24, 51].

Acute Promyelocytic Leukemia (APL), a subtype of AML, which is characterized by a block in differentiation at the promyelocyte stage, is known to be associated with at least five types of reciprocal chromosomal translocations. These translocations always involve the retinoic acid receptor-alpha (RARα) on chromosome 17. The majority of APL patients carry the chromosomal translocation t(15; 17) that fuses RARα to the promyelocytic gene PML [52-54]. The fusion genes encoding PML/RARα protein are known to inhibit the expression of C/EBPε, a transcription factor important in the terminal stages of neutrophil maturation [55] and may also inhibit C/EBPα activity [56]. APL patients which carry the PML/RARα fusion protein in their

cells respond to retinoic acid RA-induced differentiation therapy [57]. This in contrast to another type of APL patients which carry the PLZF/RARα fusion protein in their cells, these patients are not affected by therapy with ATRA as a result of its stronger association with the co-repressor proteins SMRT, mSIN3 and Histone Deacetylase-1 (HDAC) [58]. APL cells with PLZF/RARα protein may, however, respond to ATRA by cotreatment with histone deacetylase (HDAC) inhibitors [59].

A very rare congenital disorder, not related to acute myeloid leukemia (AML), is the neutrophil Specific granule Deficiency (SGD) [60, 61]. SGD is another example that supports the hypothesis that transcription factors are critical for the differentiation of myeloid cells. The neutrophils of SGD patients are blocked at the promyelocyte to myelocyte stage in their differentiation program caused by homozygous mutations of the C/EBPε gene. There are, however, only five SGD patients reported worldwide and only 2 of them show structural abnormalities in the C/EBPε gene, which probably means that the molecular basis of SGD may involve other factors as well [61, 62].

B. 1. 4. Transcription factors and neutrophil granule protein expression

A characteristic of normal neutrophil maturation is the induction of granule protein expression. Potential binding sites for a variety of transcription factors have been identified in the promoter –and/or enhancer regions of several neutrophil granule proteins.

In human, the gene encoding the early-appearing granule protein myeloperoxidase (MPO) contains a functional Sp1 site in the 139 bp minimal promoter [63] and has a distal enhancer with a functional binding site for AML1 [64]. The mouse and human MPO promoters are in close homology [65, 66], lack both a TATA-box, but transcription of the human gene is initiated near a CATAA element adjacent to the first exon [67, 68]. The distal enhancer of human MPO, which shows a functional binding site for AML1, is located 1 kb upstream from the transcription start site and shows close homology with the mouse MPO enhancer [69]. The mouse MPO enhancer is also activated by PU.1 and C/EBPα [25, 70, 71] and c-myb cooperates with PEBP2/CBF

(murine homolog to human AML1) to regulate this enhancer activity [45, 72]. The genes of proteinase 3, elastase and azurocidin, three members of the serine protease family, are clustered within 50 kb on the human chromosome 19 [73]. The promoter activity of the proteinase 3 (PR3) gene is dependent of a functional PU.1 site and a potential CG-site within the first 200 bp upstream of the transcription start. This promoter region also contains a potential TATA-box [74] and a functional C/EBP - and c-myb site [75, 76]. Studies in mouse 32Dcl3 myeloid cells revealed an enhancer located in the second intron of the proteinase 3 gene which bound Sp1 and an ets factor [77]. All the azurophil granule serine proteinase promoters contain a TATA-box [73, 78, 79]. Cis-elements found to be functional in the mouse elastase promoter include PU.1, C/EBPα and c-myb [80]. Alignment of the first 120 bp of the mouse proteinase 3 and mouse elastase promoters shows conservation of the position of the binding sites for c-myb, PU.1 and TATA-box [81].

The alpha-defensins (HNP1 and HNP3) have promoters that contain potential binding sites for c-myb, AML1, ets family members, and CDP/cut (CCAAT displacement protein) [82]. CDP is known as a repressor of transcription at the myeloblast and promyelocyte stage and its activity as repressor is down-regulated upon differentiation of myeloid cells [83-85]. However, the functional significance of this CDP-site in the HNP1 and HNP3 promoters is not yet demonstrated.

The gene expression of human lactoferrin, a secondary granule protein, is also repressed by CDP/cut (CCAAT displacement protein). Maturation past the promyelocytic stage results in decreased binding of CDP/cut to the silencer element, leading to high levels of lactoferrin mRNA expression [86]. Another transcription factor which binds to the lactoferrin promoter upon induction of maturation is C/EBPε, a transcription factor known to be critical in the terminal steps of neutrophil differentiation [87]. Uninduced human leukemic NB4 cells which do not lack C/EBPε, lack lactoferrin because of persistent binding of CDP/cut to the silencer element in the lactoferrin promoter [86]. There are many sequence similarities between human and mouse lactoferrin promoters including functional binding sites for Sp1 [88, 89] and C/EBPε [90]. A study done with nuclear extracts derived from the human monoblastic cell line U937

revealed a functional binding of C/EBPα and Sp1 to the lactoferrin promoter and it is hypothesized that both factors interact with each other to positively regulate the expression of the endogenous gene [91].

In a very recent report, C/EBPα is bound to C/EBP sites in the promoters of human lactoferrin and human collagenase in uninduced MPRO cells, which do not express the mRNAs of both secondary granule proteins, C/EBPα probably binds to an heterodimeric partner to negatively regulate these late-appearing granule proteins. In ATRA-induced MPRO cells, there is a change in the dynamics of C/EBP binding, probably resulting in C/EBPα:ε heterodimer formation, which correlates with the induced expression of the lactoferrin and collagenase genes [92]. Here, it should be remembered that all the studies are done in the mouse MPRO cell line and that regulation may be different in the human context.

The important role of C/EBPε in the regulation of the secondary and tertiary granule gene expression is based on several studies, including studies with C/EBPε -deficient mice. The granulocytes of C/EBPε -deficient mice show a block in differentiation at the promyelocyte-myelocyte stage and as a consequence these granulocytes lack secondary and tertiary granule proteins, such as lactoferrin and collagenase. Screening of the human leukemic cell lines, HL60, NB4 and U937, which are blocked in differentiation at the promyelocyte stage, showed no expression of the secondary granule proteins such as lactoferrin and collagenase [93, 94]. The ectopic overexpression of C/EBPε in U937 cells can induce the transcription of these secondary granule proteins, such as lactoferrin, and induces in parallel the differentiation of these cells [43].

B. 1. 5. Summary of transcription factors and neutropoiesis

Neutrophils and monocytes/macrophages develop from a common multipotential myeloid precursor stem cell and have some common transcription factors necessary for their differentiation. During neutropoiesis (granulopoiesis), PU.1, C/EBPα, RARα, AML-1 and c-myb are critical for the expression of the early-appearing azurophil granule proteins, while C/EBPε

plays a role in the expression of the late-appearing secondary and tertiary granule proteins. The expression of the secondary granule protein lactoferrin and the tertiary granule protein gelatinase is repressed by CDP. In **figure 3**, I present a partly hypothetical distribution scheme of neutrophil transcription factors and granule proteins during neutrophil differentiation in human bone marrow, as modified from [95, 96].

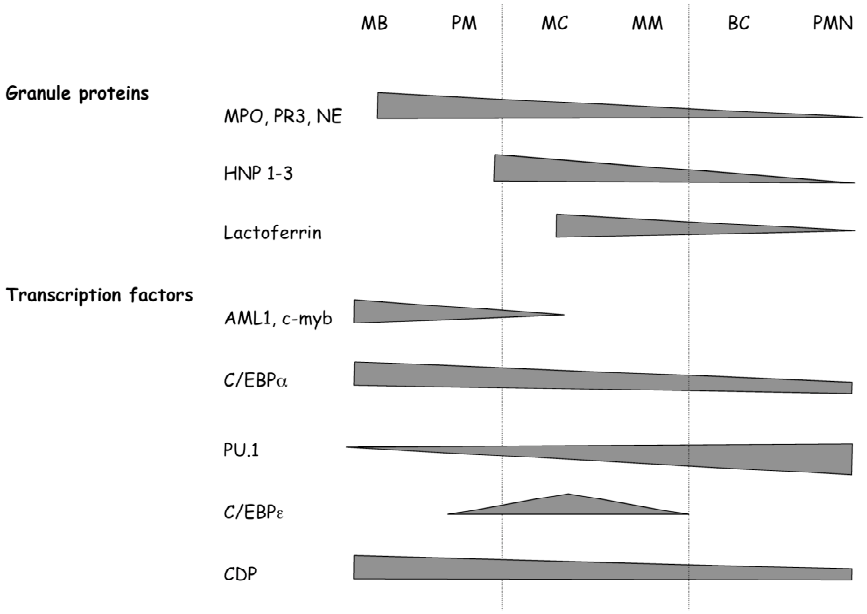

Figure 3. A partly hypothetical distribution scheme of neutrophil transcription factors and granule proteins during neutrophil differentiation in human bone marrow, as modified from Borregaard N. (2001) and Bjerregaard MD. (2003). MB: myeloblasts; PM: promyelocytes; MC: myelocytes; MM: metamyelocytes; BC: band cells; PMN: polymorphonuclear neutrophils.

What will follow is a description of protein structure, expression pattern, protein-protein interactions (cooperativity and synergy with other factors) and post-translational modifications of some transcription factors critical for myelopoiesis. Summary is given in **Table 1**.

PU.1

PU.1 is a member of the Ets family of transcription factors and binds DNA as a monomer via its C-terminal Ets domain to the consensus site 5'-AAAG(A/C/G)GGAAG-3'[97]. High levels of both PU.1 mRNA and DNA binding activity have been found in B cells, monocytes and granulocytes and the ability of PU.1 to activate its own promoter provides an explanation why there are such high expression levels of PU.1 in myeloid cells [98]. A co-activator of PU.1, called c-JUN, is required to differentiate myeloid cells towards monocytes [32] and c-JUN levels increase during monocytic differentiation [99]. C/EBPα down-regulates c-JUN and this mechanism is critical for the differentiation of myeloid cells towards granulocytes [100]. PU.1 also interacts with GATA-1, another transcription factor [101, 102], and inhibits the ability of GATA-1 to activate erythroid genes, thereby contributing to the expression of myeloid-specific genes [103, 104]. PU.1 has been shown to functionally interfere with the Retinoic Acid Receptor (RARα) and this interaction can inhibit PU.1 transactivation function, but the functional significance with respect to myeloid development of this latter interaction is not known [105]. Interferon Consensus Sequence Binding Protein (ICSBP) can interact with PU.1 in monocytic cells, enabling trans-activation via a hybrid DNA-binding element [106, 107]. Beside the physical interactions with c-JUN, GATA-1, RARα and ICSBP, the transactivating potential of PU.1 can be modulated by phosphorylation [108]. MAP kinase and JUN kinase can phosphorylate PU.1 [109] with phosphorylation of serines 41 and 45, possibly regulating PU.1 activity in myeloid cells [110].

CCAAT/Enhancer Binding Proteins (C/EBPs)

This family of transcription factors includes C/EBPα, β, γ, δ, ε and CHOP, which are all strongly similar in their C-terminal dimerization (leucine-zipper) domains and DNA-binding (basic-region) domains, but differ in their N-terminal transactivation domains, with the exception of CHOP, which lacks this transactivation domain completely [111-113]. C/EBPα is the founding member of this family and was the first protein noted to have the leucine zipper domain [114]. Because of the high conservation of the leucine zipper domain, the different C/EBP proteins are able to form homodimers with themselves or heterodimers with all the other intrafamilial proteins [115]. Both the leucine zipper dimerization motif and the basic DNA-binding motif assume an α-helical configuration and dimerization is a prerequisite for DNA binding [116-118]. C/EBPγ and CHOP lack the N-terminal transactivation domain and therefore act as inhibitors of transcription by forming inactive heterodimers with other C/EBP members [119, 120]. The C/EBP proteins cannot only form protein-protein interactions with other leucine zipper proteins, but can also bind transcription factors which do not possess this domain [121], including NF-κB, Rel-proteins [122, 123] and Sp1 [124].

By use of alternative translation initiation codons, C/EBPα and C/EBPβ can produce different sized polypeptides, also called isoforms. C/EBPα is present in isoforms 42 kDa and 30 kDa and the isoform of 30 kDa shows lower activation potential than the 42 kDa form [125]. C/EBPβ produces at least 3 isoforms: 38 kDa, 35 kDa and 20 kDa [126].

A combination of differential splicing and alternative use of two different promoters in the gene of C/EBPε gives four different mRNA isoforms, which in turn are translated into four different-sized C/EBPε proteins: 32 kDa, 30 kDa, 27 kDa and 14 kDa. Full-length protein of 32 kD possesses the greatest transactivating potential, while the 14 kDa protein lacks the transactivation domain and can inhibit the activity of the other isoforms [41]. Detailed information about the expression of C/EBPε isoforms during myeloid differentiation is still unknown, but studies have shown that the two isoforms of

32-and 30 kDa can directly bind with the DNA binding domain of c-myb and cooperatively activates the transcription of myeloid promoters [127].

The C/EBP proteins are expressed in a number of tissues, but their expression in the haematopoietic system may be limited to myeloid cells [128], with the exception of C/EBPε expression which is expressed also in lymphoid cells [55, 129, 130]. C/EBPα is besides myeloid cells, expressed at high levels in the adipose tissue, liver, intestine, lung, adrenal gland, peripheral-blood mononuclear cells and placenta [111, 131].

AML-1

This transcription factor is a member of the core binding factor (CBF) -family. CBF proteins are heterodimers composed of an α-subunit (CBFα1; CBFα2 or AML-1; CBFα3) and the β-subunit (CBFβ) [132]. The CBFβ does not bind DNA, but can interact with CBFα subunits via their Runt domains and this interaction increases the affinity for DNA of the CBFα. AML-1 binds DNA to its consensus sequence TGT/cGGT [133, 134]. AML-1 can also interact with DNA indirectly via interaction with a subset of Ets family members, including PU.1, and via the interaction with C/EBPα [48, 135, 136]. The activity of AML-1 is also regulated post-translationally; AML-1 activity is increased by phosphorylation mediated by ERK1 [137].

AML-1 is expressed in haematopoietic cells, largely restricted to myeloid and lymphoid cells, but is also expressed in nervous tissue, skeletal muscle, and reproductive tissues [33, 138, 139].

Sp1/Sp3

Promoters which lack a TATA box are often dependent on a functional Sp1 site, which can interact with the TATA-binding protein (TBP) [140]. Sp1 (Specificity Protein 1) was the first known sequence-specific regulator of mammalian gene transcription when it was identified by its ability to bind and activate GC-rich sites in the early promoter of the SV40 tumor virus [141]. Sp1 DNA binding is mediated by a characteristic pattern of three zinc fingers

located in the C-terminus of the protein. The N-terminus activates transcription, but also dimer formations and interactions with the general transcriptional machinery [142]. Several studies have identified seven additional members of the Sp family, which are referred to as Sp2-8 [142], which all can bind to GC-rich sequences. Sp3 shows more than 90% identity to Sp1 in the zinc finger DNA-binding region [143]. Some myeloid GC boxes are bound exclusively by Sp1 or Sp3, whereas others can be bound by both factors [144]. Sp1 and Sp3 are both expressed ubiquitously, and they compete for binding to GC boxes in several cell types [142]. Therefore, the ratio of Sp1:Sp3 proteins, rather than the absolute level of either protein, may affect target gene expression. Generally, Sp3 is a weaker transcriptional activator than Sp1, and Sp3 can also act as a repressor [145].

Sp1 and Sp3 are ubiquitously expressed proteins, and studies in mice indicate that Sp1 levels are highest in developing hematopoietic cells, fetal cells, and spermatids [146].

Functional cooperation of Sp1 and PU.1 in myeloid cells is in several cases necessary for maximal activity of myeloid promoters [147] and it is hypothesized that the cooperation between Sp1 and C/EBPα is essential for the promoter activity of the secondary granule protein lactoferrin [91]. As with lactoferrin expression, cooperative activity of Sp1 and C/EBPα may depend on the recruitment of transcriptional co-factors, like p300, which can acetylate histones and transcription factors, including Sp1 [148, 149]. Sp1 and Sp3 are both targets of post-translational modifications. These modifications contribute to their tissue-specific functions. Several kinases can phosphorylate Sp1, like protein kinase A [150]. Phosphorylation of the N-terminus of Sp1 increases its DNA-binding affinity, thereby increasing Sp1 transcriptional activity [151]. Reduced DNA binding and therefore, reduced transcriptional activity is found when the zinc fingers of Sp1 are phosphorylated [152]. Glycosylation of Sp1 affects its nuclear localization and has both positive and negative effects on the transcriptional activity by altering physical interactions with the basal transcriptional machinery. Glycosylation also stabilizes the Sp1 protein; low glycosylation levels appear to initiate degradation [142]. Activity of Sp1 and Sp3 is also regulated by direct acetylation. In the case of Sp1, acetylation has

various effects on the DNA binding and activity [144]. Acetylation of Sp3 is probably necessary for its repressive activity [153], but the effect of this modification on the transcriptional activity of Sp3 is still unclear. Recent work has shown that the single lysine residue in the Synergy Control (SC) motif of Sp3 is a target for sumoylation which is essential for the repressive activity of Sp3 and is in this way involved in the reduced transcriptional activity of Sp3 [154]. Sumoylation means the addition of a small ubiquitin-related modifier (SUMO) to a single lysine residue in a variety of target proteins and this binding can modulate their activity [155].

CCAAT displacement protein (CDP)

CDP is a widely expressed protein that represses gene expression via binding to a CCAAT box and displacing the binding of positively acting factors [156]. CDP is a homeodomain protein with extensive homology to the *Drosophila cut* protein [157]. Recently, it was shown that the DNA-binding activity of CDP depends on proteolytic cleavage that removes the N-terminus of CDP [158].

Name transcription factor	**Family**	**Demonstrated functional binding to target gene**	**Cooperative proteins**
PU.1	ets	MPO (mouse)[25, 70, 71]; Proteinase 3 (human)[74]; Elastase (mouse)[80]	Co-activator c-JUN; GATA-1; RAR; ICSBP
C/EBPα	Leucine zipper	MPO (mouse)[25, 70, 71]; Elastase (mouse)[80]; α-defensins (human)[82]; Lactoferrin (human)[87, 91]; Collagenase (human)[92]	Other C/EBP members; NF-κB; Rel proteins; Sp1
Onspecific C/EBP	Leucine zipper	Proteinase 3 (human)[75]	Other C/EBP members; NF-κB; Rel proteins; Sp1
C/EBPε	Leucine zipper	Lactoferrin (human & mouse)[87]; Collagenase (human)[92]	Other C/EBP members; NF-κB; Rel proteins; Sp1; c-myb
AML1	Core Binding protein (CBF)	MPO (human & mouse)[45, 64, 72]	PU.1; C/EBPα
Sp1	Zinc finger	Proteinase 3 (mouse)[77]; MPO (human)[63]; Lactoferrin (human & mouse)[88, 89]	PU.1; C/EBPα
RARα	Superfamily of nuclear receptors	C/EBPε[43]	RXR receptors
c-myb	Leucine zipper	Proteinase 3 (human)[75, 76]; MPO (mouse)[72]; Elastase (mouse)[80]	C/EBP members
CDP/cut	Homeodomain protein	Lactoferrin (human & mouse)[86]	

Table 1. Summary of transcription factors involved in myelopoiesis.

C. General aspects of transcriptional regulation

C. 1. The core promoter

The core promoter is a DNA sequence which is located immediately adjacent to and upstream of the gene and downstream of the regulatory promoter as illustrated in **figure 4**. RNA polymerase II (Pol II) and the general transcription factors (GTFs), including TFIIA; TFIIB, TFIID; TFIIE; TFIIF; and TFIIH [159], belong to the general transcription machinery. The general transcription machinery bind the core promoter which directs RNA polymerase II (Pol II) to start the transcription at the correct transcription start site. Generally, the core promoter alone is inactive *in vivo*, but *in vitro* it can bind the general machinery and activate gene expression at low or basal levels. A typical core promoter contains a TATA-box located 25-30 bp upstream of the transcription start site and binding sites for the multisubunit protein TFIID. TFIID is the only general transcription factor capable of binding the core promoter independently and specifically. The TATA-Binding Protein (TBP) together with 10 or more TBP-associated factors (TAF_{II}s) is part of the multisubunit protein TFIID and makes direct contact with the TATA-box [160, 161]. The binding of TFIID to the TATA-box makes it possible for the other general transcription factors and for RNA polymerase II to bind to the core promoter and this complex of proteins, together with coactivators, forms the RNA polymerase II holoenzyme [161, 162].

A second type of core promoter contains an initiator element instead of the TATA-box. Initiator elements are known to show similar functions as the TATA-box. The initiator element overlaps the transcription start site and binding of TFIID to this element is influenced by TFIIA and cofactors called TICs (TAF_{II}-and initiator-dependent cofactors) [163, 164]. Beside the typical core promoter structures with TATA-box or initiator element, a third class of core promoters lack both of these sequences. Instead, these promoters often contain a high G/C content and multiple sites for the ubiquitous transcription factor Sp1 [165]. At these promoters, Sp1 directs the formation of the preinitiation complex to a region 40 to 100 basepairs downstream of its binding sites.

C. 2. Regulatory transcription factors

The transcription factors which are active during myelopoiesis, as discussed above, belong to large group of so called regulatory proteins or regulatory transcription factors (RTFs). These regulatory transcription factors contain a DNA-binding domain which binds the protein to specific sequences (specific *cis*-DNA elements), including the regulatory promoter and enhancer sequences (as illustrated in **figure 4**). The regulatory promoter is located approximately 100-500 basepairs upstream from the core promoter. The enhancer sequence is found at positions up to several thousand upstream from the core promoter, but can also be localized downstream in an intron sequence of the gene. The regulatory proteins contain an transactivation domain which can initiate the gene transcription [166, 167]. The binding of the regulatory transcription factors to the regulatory promoter or the enhancer sequence is generally cooperative, which means that one transcription factor binds weakly, but multiple transcription factors which undergo protein-protein interactions with eachother give rise to increased affinities to DNA and in this way results in stronger gene transcription, also called 'synergy' in transcription [168].

The combinations of the regulatory transcription factors bound to the upstream localized enhancer sequences gives a nucleoprotein structure, called enhanceosome [169]. The enhanceosome recruits chromatin-modifying activities and interacts with the general transcription machinery and recruits it to the core promoter to form the preinitiation complex. The link between the proteins of the enhanceosome and the proteins of the general transcription machinery can be done by a mechanism called DNA-looping. Beside the looping of DNA, there are also other mechanisms that make it possible for enhancer sequences and their bound proteins to work at a distance and in this way activating the transcription. These mechanisms include nucleosomal rearrangements, done by proteins sometimes called coactivators [170, 171].

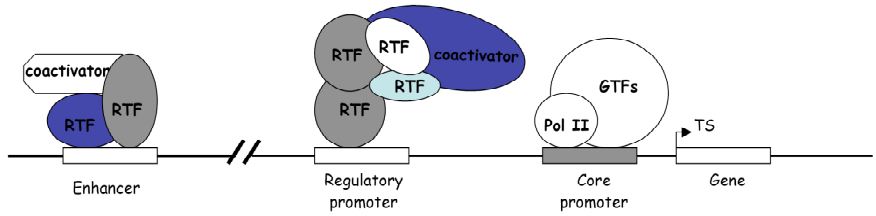

Figure 4. Schematic representation of the gene structure and the proteins involved in the initiation of gene transcription. Legend: Pol II, RNA polymerase II; GTFs, General Transcription Factors; RTF, Regulatory Transcription Factors, TS, Transcription start site.

C. 3. The activation or repression of a gene

Genes in an inactive state are tightly assembled into chromatin composed of histones, which form together a structure called a nucleosome. The histones can be modified post-translationally resulting in conformational changes of the nucleosome. Inactivated genes are often methylated and this methylation leads to binding of enzymes, called histone deacetylases (HDACs) [172]. Histone deacetylases (HDACs) are enzymes which are responsible for the deacetylation of the histone proteins of the chromatin structure and makes genes transcriptionally inactive. In contrast, the histone acetyl transferases (HATs) are enzymes involved in the acetylation of histones and has been associated with transcriptionally active genes. Remodeling of chromatin, like decondensation and acetylation of its histones, is crucial for the binding of RNA polymerase II (Pol II) and the whole preinitiation complex, because genes are not accessible for transcription factors when they are assembled into condensed or deacetylated chromatin [173]. The mammalian coactivator, p300 and its related homolog CBP, for example, are able to recruit histone acetyl transferases (HATs) [171], while corepressors like SMRT are associated with histone deacetylases (HDACs) [174-176]. During myelopoiesis for example, in the absence of the ligand retinoic acid (RA), the corepressors, like SMRT are bound with HDACs, while binding of retinoic acid (RA) to its receptor induces the release of these corepressors and induces the binding of coactivators with

HATs [177]. The latter leads subsequently to the initation of transcription of some myeloid-specific genes [38].

The activation of a differentiation-related gene is in general transient and therefore genes may be dynamically turned on and off, as for example during the differentiation program of myeloid cells in the bone marrow. The inactivation of a gene, also called repression, can, besides methylation and deacetylation, involve other types of mechanisms. First, it can occur by inactivation of a regulatory transcription factor by post-translational modifications or dimerization of the regulatory transcription factor with a nonfunctional protein or by competition for a binding site. The tagging or binding of a small protein, called ubiquitin, to the transcription factor can lead to the destruction of this transcription factor by the proteasome [178]. Secondly, repression can be mediated by inhibition of the preinitiation complex; and finally, repression can involve the binding of sequence specific repressors to silencer elements [179]. Corepressors can interact with the same DNA-binding transcription factor and in this way, a single DNA sequence can both mediate activation and repression of the transcription of a gene.

D. Proteins and peptides and their role in innate immunity

D. 1. The migration of mature leukocytes to sites of infection

The polymorphonuclear neutrophils (PMNs) and monocytes/macrophages play a central role as phagocytic cells in the first line of defense and are important components of the innate immunity [180] [5, 181]. Following release from the bone marrow into the blood circulation, mature neutrophils are incapable of cell division, stay very shortly (about 6-10 hours) in the circulation and subsequently migrate into the tissues, where they undergo spontaneous apoptosis after 1-2 days [2, 182]. In response to inflammatory stimuli, neutrophils become activated and migrate from the circulating blood to infected tissues, where they efficiently bind, phagocytoze, and kill bacteria and other invaded microbes [183, 184]. Monocytes can also be activated upon stimulation

of inflammation, they migrate and differentiate into macrophages in the infected tissues and play their roles as long-lived (several months or even years) phagocytes [1, 184].

The migration of leukocytes (or phagocytic cells) from the circulating blood to sites of infection in the extravascular tissues involves a series of sequential events regulated by several molecular interactions [185, 186]. The first steps in this migration process involve tethering to and rolling of the leukocyte along the endothelial cells of the vessel wall. This slowing down of leukocytes is mediated by weak and reversible molecular interactions between the selectins of the leukocytes and endothelial cells [185, 186]. Other inflammatory factors (chemokines, cytokines), such as platelet-activating factor (PAF), IL-8, fMLP, TNF-α, IL-1β, and LPS, bind to receptors on the cell membrane of the phagocytic cells and activate them to express integrins on the cell surface [187]. Integrins, together with selectins, make the phagocytic cells tightly adherent to the endothelial cell wall. After shape change and polarization, the leukocytes migrate through the endothelial cell wall, penetrate the basal membrane, the extracellular matrix of the connective tissue to reach the infected extravascular tissue.

D. 2. Antimicrobial proteins and peptides in phagocytic and non-phagocytic cells

Killing and destruction of microorganisms after phagocytosis is mediated by an NADPH- dependent oxidase that transfers electrons from NADPH to oxidase (O_2) to form oxygen radicals (O_2^-), a reaction called 'respiratory burst' [188]. Oxygen radicals are converted to hydrogen peroxide (H_2O_2) which with the help of the azurophil granule protein myeloperoxidase (MPO) is converted to hypochlorous acid (HOCl), the most bactericidal agent produced by the neutrophil [189]. Macrophages also possess this oxygen-dependent mechanism to kill microorganisms [190], but do also, together with the neutrophils, contain a wide range of oxygen-independent antimicrobial proteins and peptides [191, 192]. Antimicrobial proteins and peptides of the neutrophil are largely stored in the azurophil and specific granules. The specific granules release their contents

both into the phagosome and into the extracellular environment, while the azurophil granules degranulate into the phagosome, where the ingested or phagocyted microorgansims are directly exposed to high concentrations of granule contents. Antimicrobial agents in the neutrophil granules may also be released into inflammatory fluids following necrosis of the neutrophil [192].
Neutrophil granule proteins with antimicrobial activity include lactoferrin [193-195]; BPI (see next section), proteinase 3 together with other serine proteases [196, 197]. The antimicrobial peptides belong to two main peptide families: the defensins and the cathelicidins [198-200]. These peptides are not only found in phagocytic cells, but are also produced in various types of epithelial cells and nonphagocytic cells [201]. Defensins are of two main classes: the α-defensins and the β-defensins. Of the six known human α-defensins, four (human neutrophil peptide, HNP-1-4) are expressed primarily by neutrophils, but they are also expressed in specific lymphocyte and monocyte subpopulations [202]. The other two, HD-5 and –6, are expressed by intestinal Paneth Cells [203, 204]. In humans, four types of β-defensins have been identified (HBDs 1-4). HBD1 and 2 are mainly synthesized by epithelial cells, but expression of HBD1 and 2 mRNA occurs also in monocytes and monocyte-derived macrophages [204, 205]. Among rodents, mice lack leukocyte defensins [206], rats have them [207], and both species have numerous Paneth cell defensins (called Cryptdins in mice) and epithelial cell β-defensins [208-211].

The combined action of oxygen-independent antimicrobial proteins and peptides results in an attack of the microbial membrane and/or cell wall, leading to growth arrest and death of the invaded microbes. And, in some cases, like for BPI and lactoferrin, the binding to lipopolysaccharide at the outermembrane of gram negative bacteria leads to neutralization of this endotoxin (LPS) [195, 212].

D. 3. Human BPI

Human BPI, a 55-kDa protein, was isolated from polymorphonuclear neutrophils (PMNs) in 1978 by Elsbach, Weiss and colleages [213], but it took almost 20 years until the crystallized structure of the full-length recombinant human BPI (Bactericidal/Permeability-Increasing protein) was studied [214]. The BPI gene is localized to chromosome 20 [215] and is composed of 15 exons ranging in size from 43 bp (exon 12) to 358 bp (exon 15) with 14 introns ranging in size from 0,6 kb (intron 8) to 6,6 kb (intron 5) [216]. Cloning and sequencing of BPI cDNA has revealed a predicted protein structure with a basic, hydrophobic N-terminal and an acidic, hydrophilic C-terminal. Together with the α-defensins (HNP 1-4), proteinase 3 and azurocidin, BPI is stored in the cytoplasmic azurophil granules of PMNs. BPI may with its hydrophobic C-terminal domain be attached to the membrane of the azurophil granules and to the cell membrane of the neutrophils (PMNs) [10, 217].

BPI is also present in lower amounts in specific granules of human eosinophils [218]; on the surface of human peripheral blood monocytes [219]; in human mucosal/colon epithelial cells [220] and in human dermal fibroblasts [221]. The expression of BPI in epithelial cells was, however, only detectable upon stimulation with inflammatory stimuli [220, 221].

BPI shows activity against gram-negative bacteria and does this in three ways: (1) it shows a cytotoxicity via sequential damage to bacterial outer and inner lipid membranes [222]; (2) it neutralizes the gram-negative bacterial surface lipopolysaccharide (LPS) or endotoxin [223] and (3) it opsonizes bacteria to enhance phagocytosis by neutrophils [224]. See **figure 5** for a schematic overview. Binding of BPI to the surface of gram-negative bacteria involves electrostatic interactions between the cationic N-terminus of BPI and the negatively-charged phosphate groups of LPS [225]. The cytotoxic effects of BPI on gram-negative bacteria are synergistically enhanced by members of the defensin family [226].

It was reported that intracellular BPI content is 3-to 4-fold higher in adult neutrophils than in newborn neutrophils and this may contribute to the increased incidence of gram-negative sepsis among newborns. However, other

studies show that only 40% of newborns have a BPI-deficiency, while others have normal levels of intracellular BPI [227].

BPI is also a target antigen of anti-neutrophil cytoplasmic autoantibodies (ANCA). The presence of anti-BPI autoantibodies (BPI-ANCA) was first detected in patients with Wegener's Granulomatosis, but are also found in diseases such as inflammatory bowel disease, ulcerative colitis and cystic fibrosis. This BPI-ANCA may make the inflammation worse by reducing the ability of BPI to destroy the gram-negative bacteria and to neutralize bacterial LPS. BPI-ANCA is associated with a higher inflammatory disease activity and greater organ damage [228].

In a randomized clinical trial, the treatment of patients having meningococcal sepsis with recombinant 21 kDa N-terminal BPI fragment ($rBPI_{21}$) has been shown to be beneficial in decreasing complications of meningococcal disease, such like necrosis which can lead to amputations [229].

D. 4. Similarities and differences between LPS-binding protein and BPI

The LPS-binding protein or LBP is constitutively secreted into normal plasma by the liver and its expression can be induced during an early phase of a gram-negative bacterial infection, also called the acute phase response. LBP transfers LPS to CD14 on the cell surface of monocytes/macrophages [232, 233]. CD14 can, via MD-2, activate the Toll-like receptor-4 (TLR-4) that in its turn transduces a signal leading to the secretion of pro-inflammatory mediators, such as TNF-α and IL-1β, which trigger the inflammatory reaction [234, 235]. See **figure 5** for a schematic overview.

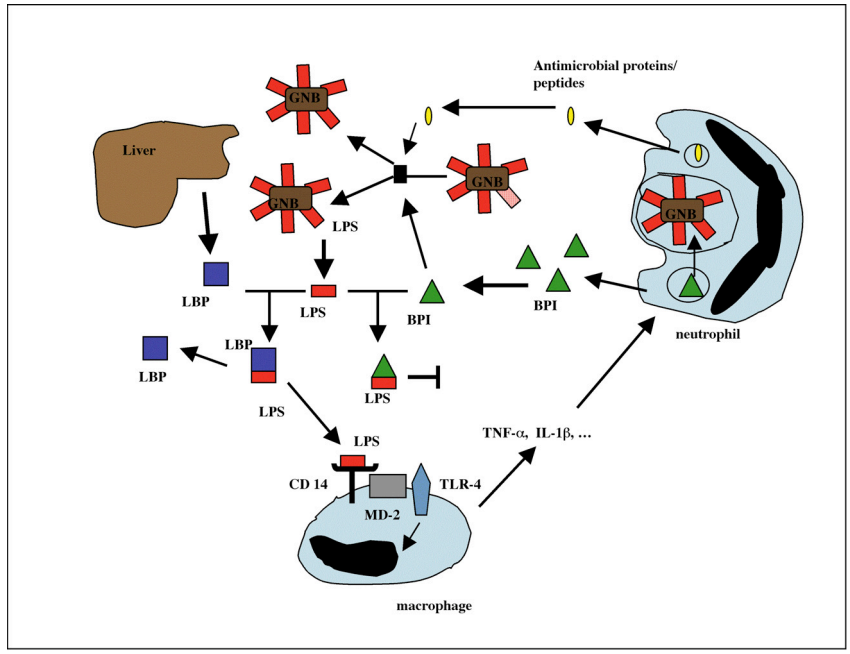

Figure 5. Modified from Levy O., 2000. GNB: gram-negative bacteria; BPI: Bactericidal/Permeability-Increasing protein; LBP: LPS-Binding Protein; LPS: lipopolysaccharide.

BPI (Bactericidal/Permeability-Increasing protein) and LBP (LPS-binding protein) bind both the lipid A [212, 236], but BPI function as an antagonist of the LBP-LPS interaction [237]. At sites of infection, a molar excess of BPI is present and here can BPI fulfill its functions extracellularly by binding to LPS leading to the neutralization of the endotoxic effect [223]. BPI and LBP are related proteins [238] encoded by genes localized in a cluster on human chromosome 20 [215] and the genomic structure (exon-intron organization) of BPI and LBP shows strong sequence homology to each other, implying a common origin of these genes [216]. Recently, BPI and its relative LBP was also found to be expressed in epithelial cells [220, 221, 239], which are not only known as mechanical barrier-cells, but are also found to have a secretory function in the first line of defence in the host [240].

D. 5. BPI and its relatives

BPI (Bactericidal/Permeability-Increasing protein) and LBP (LPS-binding protein) belong to a related protein family, including CETP (Cholesteryl Ester Transfer Protein) and PLTP (Phospholipid Transfer Protein) [241]. BPI and LBP, as described above, function as antagonists in the innate immune response against gram-negative bacteria. The two other proteins, CETP and PLTP are produced by the liver and function in the transport of lipids between lipoprotein particles in the bloodstream and do not have any known function in the innate immune system [242]. BPI, LBP and PLTP are encoded by related genes located on chromosome 20, while the CETP gene is located at chromosome 16 [241].

A novel family of secreted proteins, named PLUNC (palate, lung and nasal epithelial clone) proteins, was recently identified and these novel proteins are expressed in the upper airways (respiratory tract), nose (nasal), and mouth (oral) [243, 244]. The family of PLUNC proteins is encoded by related genes localized in a cluster on human chromosome 20 [243] and in a cluster on mouse chromosome 2 [245]. The proteins of the PLUNC family show very low primary amino acid sequence identity (below 40%), but are predicted to be structurally similar to BPI and LBP [214, 244]. Based on its expression pattern and the structural similarities with BPI and LBP, it is suggested that PLUNC proteins play a potential role in the innate host defense, but the exact function is still not known [241]. The mouse orthologs of the human PLUNC proteins are specifically expressed in the tongue and minor salivary glands and the mouse PLUNC family includes PSP (parotid secretory protein), VEMSGP (von Ebner minor salivary gland protein), SPLUNC1, and SPLUNC5 [246]. Because of the identification of many new PLUNC-genes in different species, PLUNC was renamed as SPLUNC1 [247], which not should be mixed up with the subdivision of the 'old' PLUNC protein family into two subgroups, the short PLUNC (SPLUNC) and the long PLUNC (LPLUNC), based on the primary sequence length and the structural predictions [244, 247]. The short PLUNC proteins (≈ 200 amino acids) show similarity only with the N-terminal domain of BPI, whereas the long PLUNC proteins (≈ 400 amino acids) are similar to both the N-and C-terminal domains of BPI and LBP [244]. Another newly

identified family of proteins related to BPI and PLUNC are the RY proteins. These RY proteins are also suggested to have potential functions in the innate immune system and show conservation between human and mouse [248].

D. 6. Proteinase 3 and its role in Wegener's Granulomatosis (WG)

Proteinase 3 (PR3), also called myeloblastin [249], is a 29-kDa serine protease stored in the azurophil granules of the polymorphonuclear neutrophils (PMNs) and in the granules of monocytes [250]. Besides azurophil granules, PR3 is also stored in specific granules, secretory vesicles and is present on the plasma membrane of resting and unactivated neutrophils [251, 252]. During phagocytosis, PR3 has an antimicrobial activity, similar to the activity of other serine proteases, cathepsin G and neutrophil elastase (NE), and this activity is both against gram-negative - and gram-positive bacteria, as well as against fungi [197, 253]. Like neutrophil elastase (NE), PR3 may play a role in the migration of neutrophils through the basal membranes and is able to degrade a variety of extracellular matrix proteins and basal membrane proteins [197, 254]. PR3 may also be involved in processing of inflammatory mediators such as processing of cytokines like TNF-α [255] and IL-8 [256] and it induces the production of IL-8 by endothelial cells [257].

PR3 is the main target antigen of antineutrophil cytoplasmic autoantibodies (ANCA) observed in Wegener's Granulomatosis (WG) [258-260]. Wegener's Granulomatosis (WG) is an autoimmune chronic inflammation that appears in several organs (kidney, nose, and lung), therefore also called 'systemic' vasculitis and is characterized by chronic inflammation of the small blood vessels, leading to necrosis in the organs which are supplied by the damaged vessels [261]. The antineutrophil cytoplasmic autoantibodies (ANCA) are strongly associated with small-vessel vasculitis or Wegener's Granulomatosis (WG), but the exact role of these autoantibodies is not yet known [262]. Increasing evidence indicate, however, that PR3-ANCA are involved in the pathogenesis of WG [263]. Not only PR3-ANCA, but also antibodies specific against myeloperoxidase, and other granule proteins, such as BPI, are found in patients with systemic small vessel vasculitis [264-267].

The membrane expression of PR3 is heterogeneous for circulating (resting) neutrophils of healthy persons and defines two neutrophil populations: with and without PR3 surface expression, respectively [252]. The proportion of $PR3^+$ neutrophils and $PR3^-$ neutrophils varies among individuals, is stable over prolonged time and is genetically controlled [252, 268]. In WG patients, an increased percentage of $PR3^+$ neutrophils and an elevated level of PR3 expression as compared with healthy individuals was found and level of PR3 expression was correlated to increased relapse rate [269]. This observation has led to the hypothesis that an increased number of $PR3^+$ neutrophils and increased amount of PR3 molecules on neutrophil membrane could be a risk factor and could play a role in the pathogenesis of WG [270].

The observation that inactivated neutrophils show PR3 expression on their membranes is controversial to other studies which only found PR3 expression on the surface of primed or pre-activated neutrophils [271, 272] and Yang *et al* argues that the neutrophil isolation procedures itself cause an *'in vitro'* stimulation which explains that other researchers, like Halbwachs-Mecarelli *et al*, found PR3 expression on the plasma membrane of resting neutrophils.

A mechanism which possibly could explain the role of PR3-ANCA in the pathogenesis of WG is as follows: Priming or pre-activation of neutrophils with pro-inflammatory cytokines, such as TNF-α, induces the translocation of PR3, and probably also other target antigens, such as MPO, from the cytoplasm (stored in granules or vesicles) to the plasma membrane, where these antigens are accesible to ANCA [272, 273]. Binding of ANCA to PR3 (or to the other target antigens) triggers signaling events that lead to neutrophil activation, causing an increase in respiratory burst (NADPH-oxidase activity), but also degranulation with the release of more PR3 and other serine proteases and production of IL-1β. These effects of the neutrophil activation leads then to damage of the endothelial cells of the vessel wall [274, 275]. It is generally assumed that ANCA interacts with PR3 via the Fab region of the antibody and its Fc region interacts with the immunoreceptor FcγRIIa on the neutrophil cell membrane, leading to the cross-linking of PR3 to FcγRIIa [276, 277]. FcγRIIa is a transmembrane protein with a tyrosine-based activation motif in its cytoplasmic domain [278] and the process of cross-linking to PR3 lead in its

turn to the activation of intracellular signaling pathways which induces the respiratory burst (or increased expression of NADPH-oxidase). The exact signaling mechansims are not fully known yet, but some studies indicate a tyrosine kinase dependent pathway, involving PI3-K, PKB/Akt and ERK [279-281], but it may also occur via a tyrosine kinase independent pathway, involving PKC [282]. Integrins in the plasma membrane of neutrophils may also be activated by ANCA and increase the adhesion of the activated neutrophils during vasculitis [283]. Another hypothesis is that ANCA antigens are expressed on the surface of apoptotic cells, get then phagocytized by macrophages, which in their turn become hyperactivated, leading to increased secretion of pro-inflammatory cytokines and may play a role in the amplification of the inflammatory proces in WG [271, 284]. This hypothesis, leads us further to the potential role of monocytes in the pathogenesis of WG [285]. One study suggests that pro-inflammatory cytokines induces translocation of intracellular stored PR3 to the membrane of monocytes, makes them accessible to ANCA, with consequences similar to those for neutrophils [272]. Recently, Ohlsson *et al* [286] found an increased PR3 mRNA expression in peripheral monocytes from patients with AASV (ANCA-associated small vessel vasculitis) as compared to healthy persons. However, this increase in PR3 mRNA could not be explained by the induction of pro-inflammatory cytokines, as suggested in previous studies [287, 288]. Yang *el al* found not only an increase of PR3 mRNA in monocytes, but also in neutrophils of patients with AASV [289].

II. Aims of the present studies

The four different studies I-V are numbered according to papers I-V, and the specific aims are as follows:

Study I:

- To identify the transcription start site of the human BPI gene in myeloid cells.
- To clone the proximal promoter of the human BPI gene.
- To identify functional elements within the cloned promoter which play important roles in the transcriptional regulation of human BPI gene during myelopoiesis.

Study II:

- To investigate whether BPI is structurally and functionally conserved between humans and mice.
- To study the expression profile of the mouse BPI and to compare the expression levels in different tissues between humans and mice.

Study III:

- To identify the transcription start site of the mouse BPI gene in myeloid cells.
- To clone the proximal promoter of the mouse BPI gene.
- To characterize the cloned promoter region for potential cis-elements and compare these elements with those found within the human BPI promoter

Study IV:

- To investigate if the SNP (Single Nucleotide Polymorphism) which introduces a new potential Sp1 transcription factor binding site may be responsible for the observed up-regulated expression of proteinase 3 (PR3) in myeloid cells of Wegener's Granulomatosis patients.

III. Experimental considerations

A. Determination of the transcription start site

Localization of the proximal promoter region can be done by determination of the transcription start site, because the promoter region is by definition located close to and upstream of the transcription start site [172]. 5'RACE, which means 5'Rapid Amplification of cDNA ends, is a technique used to localize the transcription start site by identification of the 5' end of the encoded mRNA. A potential risk of this technique, however, is degradation of mRNA leading to inaccurate determination of the transcription start site. Therefore, it was of interest to also use an alternative to the conventional 5'RACE, namely the 5'RML-RACE technique. In 5' RML-RACE, the poly (A) mRNA is first treated with Calf Intestine Alkalin Phosphatase (CIP) to remove all the free 5' phosphates from degraded mRNA, ribosomal RNA and contaminating genomic DNA. The cap structure, which is found on intact or full-length mRNA protects intact mRNA from CIP, but is removed by Tobacco Acid Pyrophosphatase (TAP), which in its turn leaves a monophophate at the 5' end of the mRNA. The next step, which is in common with the first step in the conventional 5' RACE method, is the ligation of an adaptor to the mRNA. In 5' RML-RACE, this ligation can only take place on intact mRNA with its 5' monophosphate and not on dephosphorylated sample material. Both methods, 5' RACE and 5'RML-RACE, will transcribe the adaptor-ligated mRNA to cDNA, which will be subsequently amplified in two nested PCR reactions using specific PCR primers which are complementary with the known cDNA sequence of the gene of interest.

A good starting point when using both 5' RACE methods, is to use cells or cell lines which express high levels of the specific transcript of interest. Human mononuclear bone marrow cells and the human promyelocytic cell line HL60 express high levels of BPI mRNA, which was an advantage when using 5'RACE. For the mouse promyelocytic MPRO.C1 cell line, I was forced to first treat the cells with ATRA to reach mRNA levels of BPI which were high enough to proceed with 5' RACE. I also tried to use

mRNA extracted from the ATRA-treated 15P-1 cells (mouse Sertoli cell line) for 5'RACE, but this trial was unsuccesful, probably because 15P-1 cells express much less of the specific BPI transcript, as compared to the MPRO.C1 cells, although 15P-1 cells respond in a similar way to ATRA as MPRO.C1 cells (**Paper II**).

A complementary method to 5'RACE is primer extension analysis, which was not used in this thesis. By using primer extension, only two specific reagents are required: a mRNA sample and a radiolabeled oligonucleotide primer which can bind complementary to the specific mRNA sequence. The resulting radiolabeled cDNA is then analyzed on a denaturing polyacrylamide gel electrophoresis and the size of the band detected on the gel, as compared with a molecular weight marker, gives us a determination of the distance between the site where the primer is bound, and the beginning of the mRNA transcript. Primer extension is less sensitive than 5'RACE, because it is not a PCR-based method, but is is more quantitative than 5'RACE and can therefore give us an idea about the level of transcriptional activity in the investigated cells or cell lines.

A disadvantage of the primer extension analysis is that it does not provide sequence information regarding the transcript. Therefore, no alignment to genomic DNA can be performed. If the primer not binds in the sequence corresponding to exon 1, the transcription start can be inadequately determined to be localised in intronic sequences. 5'RACE, on the other hand, provides sequence information on the transcript and therefore in this respect is superior to primer extension analysis.

B. Measurement of transcriptional activity

The promoter strength (or level of promoter activity) is generally considered to be proportional to the number of specific mRNA transcripts synthesized per time unit by the RNA polymerase II [172]. An indirect way to measure promoter activity is the reporter assay in which determination of enzyme levels is used as an indirect measurement of promoter activity. In this thesis, I used the luciferase reporter assay in which the promoter region of interest

is inserted immediately and upstream of the *firefly* luciferase gene in a so called reporter vector. The inserted promoter is thus able to activate the transcription of the *firefly* luciferase gene and the translated *firefly* luciferase enzyme will then catalyze a reaction resulting in light emission, which then finally can be measured in a luminometer. The most common way, and also the one I used, to introduce the vector into cells is by performing transient transfections. In a transient transfection procedure, the reporter vectors which contain the promoter region of interest are transfected into cell lines and within a short period, typically 10 to 24 hours, but sometimes longer, the enzyme activity is measured. Transient transfections are generally performed in cell lines which express the endogenous gene from which the promoter region is derived. The human BPI promoter showed highest activity in HL60 cells which also were found to express a relative high level of the endogenous BPI gene. The human cervix carcinoma cell line HeLa can be used as a negative control for specific promoter activity, because this cell line did not express detectable amounts of any BPI mRNA transcript. To compare promoter activity between different cell lines, for example between U937 cells and HL60 cells, normalization of specific enzyme activity levels to levels obtained by transfection of control reporter vectors is necessary. A negative control is a transfection with the promoterless pGL3 basic vector, which is also used for insertion of the promoter region of interest. Positive control transfection is performed with a vector containing a strong viral promoter fused to the *firefly* luciferase gene, such as pGL3 promoter. Both vectors, pGL3 basic and pGL3 promoter, are used in this thesis. The level of enzyme activity obtained by transfection of pGL3 promoter gives us an idea about the relative strength of our promoter of interest in the individual cell lines.

To compensate for varying transfection efficiencies between different experiments, internal controls can be used. In our studies, the *Renilla* luciferase reporter vector was used as internal control and was co-transfected with the *firefly* luciferase reporter vector. The *firefly* and *Renilla* luciferases are of distinct evolutionary origins and have different enzyme structures and substrate requirements [290] and it is therefore possible to

specifically determine each of their activity when co-transfected into the same cell population [291].

By using stable transfection assays, which is an alternative to transient transfection assays, the vector containing the reporter gene and the promoter region of interest can be stably integrated in the genome of the transfected cell line. The advantage of the stable transfection assay as compared to the transiently, is that the analyzed promoter is surrounded by a more natural chromatin configuration and at a more natural copy number [292]. A disadvantage is of course that it can be inadequately affected by surrounding promoter and enhancer sequences.

In addition to the indirect reporter assay, a direct way to measure transcriptional activity is to measure the steady-state mRNA levels of the endogenous gene of interest. The steady-state mRNA level is defined by the level of mRNA as a result of its rate of synthesis and its rate of degradation [172]. I have been using three methods in this thesis which are in common to measure the steady-state mRNA levels in cells and tissues. These three methods include Northern blot, the standard RT-PCR and the fluorescence-based real time RT-PCR. Northern blot analysis is based on the gel electrophoresis of total RNA samples and specific mRNA is detected by using labeled oligonucleotide, cDNA or RNA probes. Northern blot analysis is the only method of all three used methods, which gives information about mRNA-size. The second method, the standard RT-PCR (Reverse Transcription - Polymerase Chain Reaction), is a rapid and extremely sensitive way of analyzing whether a gene is expressed or not and can also provide a semi-quantitative information about steady-state mRNA expression levels. RT-PCR is a PCR-based method and is much more sensitive than Northern blot. RT-PCR needs less than 1μg of total RNA or 10-100 ng mRNA, while Northern Blot needs 2-20 μg of total RNA. In RT-PCR, the RNA template is first transcribed into cDNA, followed by its amplification in a standard PCR reaction using sequence-specific primers. The end-products of this reaction are then analyzed by standard agarose gel electrophoresis.

A PCR amplification typically proceeds in three phases, an early slow phase where initial products are formed; a mid phase, in which products are amplified in an 'exponential' manner; and a late phase, were product amplification reaches a plateau, which also involves the end of the PCR reaction. The plateau is reached as a consequence of changes in relative concentrations of certain components of the reaction and therefore some PCR reactions will generate more product than others. In some PCR reactions, the exponential phase may start later, in which more cycles are often needed in order to reach the plateau phase [293]. It is because of these limitations that the analyzing of the end-product in the standard RT-PCR is unreliable as a way to quantitatively analyze differences in steady-state mRNA levels between different cells or tissues.

Therefore, in a third method, the fluorescence-based real time RT-PCR, it is possible to measure the amount of PCR product at a point in which the reaction is still in the exponential phase. During the exponential phase in real time PCR reaction, a fluorescence signal threshold is determined at which point all samples can be compared. This threshold is called cycle threshold, or C_t , and is defined as the number of PCR cycles required to generate enough fluorescence signal. The C_t value is directly related to the amount of RNA template and is therefore the basis for calculating steady-state mRNA expression levels [294]. In the mouse BPI ortholog study, relative expression levels of steady-state mRNA were determined by calculating the differences in the C_t values (ΔC_t) of the genes of interest before and after treatment with ATRA. The expression level of the house-keeping gene β_2 –microglobulin was used as normalization before calculating the relative expression dfifferences. Due to the exponential nature of real time PCR, the ΔC_t is converted to a linear form by $2^{-(\Delta Ct)}$.

In this thesis, we only measured transcriptional activity by an indirect reporter assay and by measuring steady-state mRNA expression levels, but it could also been of interest to consider the analysis of transcriptional activity by measurement of the *de novo* RNA expression. The nuclear run-on assay, for example, is a method based on the mearument of the *in vivo* rate of transcription initiation from a specific gene. Briefly, isolated cell nuclei are

mixed with radioactive NTP to label the DNA-bound polymerase molecules and the amount of specific radiolabeled pre-mRNA is than measured by autoradiography [172]. The nuclear run-on assay shows results that are not influenced by mRNA degradation and can give a strong indication if the expression of a gene is regulated at the level of transcription or at the level of mRNA stability. In general, gene expression is not only regulated at the level of transcription, but can also be controlled by the intrinsic stability of mRNA. The amount of time required for 50% of the mRNA to degrade is called half-life and this can be short or long for a given mRNA. Many studies are available to measure mRNA stability or degradation, including the inhibitor studies with a substance called actinomycin D, which can enter the cells and can block transcription. The amount of intact mRNA remaining at various time points after addition of actinomycin D can be analyzed and gives an answer about the half-life of the mRNA [172].

If the promoter activity, as indirectly measured by luciferase activity, is strong and in good correlation with the steady-state mRNA expression levels found by real time RT-PCR, standard RT-PCR or Northern Blot, the gene is most likely regulated at the level of transcription.

C. Determination of the functional importance of transcription factors

C. 1. Deletion analysis

To get better understanding about the transcription factors which are functionally involved in the regulation of the endogenous gene expression, we analyzed the promoter region of interest in a first step by deletion analysis. Deletions of a promoter region can be made by PCR, but also by using the enzyme Exonuclease II, and the resulting deletion mutants, which can be five or more different-sized promoter fragments, are then inserted in the luciferase reporter vector, respectively, and tested for promoter activity by performing transient transfections.

C. 2. Computer-based search for potential cis-elements

The next step in the investigation of the functional importance of transcription factors (trans-elements) in the regulation of the expression of the endogenous gene, is the identification of binding sites (cis-elements) in the cloned promoter region of interest. We identified potential cis-elements within the sequence of the deletion mutants with highest promoter activity by using a Transfac database. Many different internet-based search programs are available to screen promoter regions of interest for possible functionally relevant cis-elements. The binding sites for transcription factors are in general short, 5 to 12 basepairs, and sequence variation within one functional binding site occurs and as a consequence many different transcription factors, from different families or within the same family, can bind to overlapping consensus sequences. In other words, one consensus sequence found in the Transfac database can be potentially recognized by different transcription factors.

Although it is impossible to completely rely on database findings, the search for potential binding sites in databases is interesting to get some information about which types of transcription factors that possibly can play a role in the regulation of a gene of interest. For example, the database search shows transcription factors which are already known to play a role in the regulation of other related genes and therefore may be involved in the regulation of the expression of the investigated gene.

C. 3. Site-directed mutagenesis

Based on the findings of some potential binding sites in the promoter region by Transfac database search, we made a selection of some interesting binding sites and analyzed these individual sites by subsitution analyzis prepared by site-directed mutagenesis. In site-directed mutagenesis, 3 to 5 nucleotide basepairs within a consensus sequence are replaced by 3 to 5 other nucleotide basepairs by PCR and the mutated promoter is then inserted in the luciferase reporter vector and tested for activity in transient transfections assays. If the mutations reduce the activity of the promoter region, the results suggest that the individual binding site play role in the activation of the promoter region, but is still not an

answer if these individual sites also play a role in the transcriptional regulation *in vivo*.

C. 4. EMSA

The identified, but still potential, transcription factors can then in a next step be analyzed in Electrophoretic Mobility Shift Assay (EMSA) to reveal if these proteins are able to bind to the specific DNA sequence in the promoter region of interest. EMSA is an *in vitro* assay and is based on the principle that a ^{32}P-labeled DNA probe containing the potential specific binding site is incubated with nuclear extracts containing DNA-binding proteins. The resulting protein-DNA complexes are separated from the free (unbound) DNA by electrophoresis through a nondenaturing polyacrylamide gel as these complexes migrate more slowly than the free DNA probe. An image of the gel is used to detect the positions of the free and bound DNA probe and the protein-DNA complex results then in a specific shift on the gel image [295]. The identification of the specific transcription factor bound to the DNA probe can be done by addition of a specific antibody to the protein-DNA complex. This latter complex migrates more slowly than the complex without antibody and the image of the gel shows a so called supershift.

Also here, in EMSA, nucleotide substitutions can be made in the radiolabeled DNA probe to see if this mutagenesis leads to the disruption of the protein-DNA complex. To get more evidence for the specificity of the binding to the DNA, competition experiments can be performed, where the radiolabeled wild-type DNA probe is incubated with increasing concentrations of unlabeled wild-type and mutant DNA probes. The specific shift of the radiolabeled protein-DNA complex decreases in the presence of increasing concentrations of the unlabeled wild-type DNA probe, because the amount of DNA-binding proteins bound to the unlabeled wild-type probe is higher than for the labeled probe. In contrast, in the presence of the unlabeled mutant DNA probe, the specific shift of the radiolabeled protein-DNA complex does not disappear or do not decreases.

EMSA is a very sensitive method which not only can reveal the binding of a specific transcription factor to its binding site, but also gives an answer if the specific transcription factor is present in high or low concentrations in the

extracted nuclei. Beside its advantages of being a quantitative and semi-quantitative method, EMSA is an assay performed *in vitro* and reveals therefore not if the protein-DNA binding is also functionally relevant *in vivo*. But, if the nucleotide subsitution made in the DNA probe in EMSA is similar to the nucleotide subsitution made in the promoter fragment in the indirect reporter assay, there is a strong evidence that the disruption of the protein-DNA complex is correlated with the reduced promoter activity and that the analyzed and specific binding site is also functional *in vivo*.

C. 5. ChIP

As a complement to EMSA, an *in vivo* assay, which we didn't use in this thesis, called Chromatin Immunoprecipitation (ChIP), and can be performed to study which cis-elements are functional important in the nucleus of the living cells [296, 297]. The basic principle of ChIP is as follows, living cells growing in their medium are cross-linked by addition of formaldehyde and for the extraction of the fixated protein-DNA complexes, cells are lysed. These fixated protein-DNA complexes are then immunoprecipitated using specific antibodies against the potential DNA-binding protein. After washing and eluting of the immune complexes, the cross-links between protein and DNA are disturbed and the immunoprecipiated DNA is recovered and used as template in a standard PCR reaction using sequence-specific primers overlapping the potential binding site. As negative control, DNA precipitated without specific antibody is used as template in the same PCR reaction. PCR products are analyzed on a standard agarose gel and a band appears when the specific antibody has bound to the DNA-binding protein of interest. The disadvantage of this *in vivo* ChIP assay is its extreme sensitivity of the PCR which makes that there is an increased risk for nonspecific precipitated DNA even in the negative control without addition of specific antibody. The sequence-specific primers in the PCR reaction can also anneal to onspecific precipitated DNA in the negative control, so an optimalization of the PCR conditions is always necessary here (e.g. cycle number).

C. 6. Overexpression of recombinant transcription factor

To test further the functional importance of the transcription factors for the regulation of the endogenous gene expression, a correlation can be made between results obtained by EMSA and those obtained by the ectopic expression of the DNA-binding protein of interest together with the reporter vector in transiently transfected cells. The choice of the transcription factor to be overexpressed was done from the site-directed mutagenesis studies and the functional binding studies in EMSA. If the overexpression of the specific transcription factor gave an effect on the promoter activity in the transfected cells, one could make the suggestion that the transcription factor is also involved in the *in vivo* promoter activation. We also analyzed the effect of the overexpression of a transcription factor on the steady-state mRNA expression of the endogenous gene. If an induced or repressed steady-state mRNA expression was demonstrated as compared to control mRNA levels, we could conclude that the transcription factor had a negative or positive effect on the expression of the endogenous gene.

Although all the methods used in this thesis to investigate the functional importance of some interesting transcription factors were performed *in vitro,* the combinations of all these methods can give results that suggest that the transcription factors are involved in the regulation of the endogenous gene at the transcriptional level.

IV. Results and general discussion

A. The BPI promoters

A.1. Are the promoters of the human – and mouse BPI genes comparable? **(Paper I, II, and III)**

The binding of transcription factors to specific regulatory sequence elements is one important mechanism for regulation of granule protein expressing during myelopoiesis [95].

In **paper I**, we set out to identify regulatory cis-elements important for myeloid-specific expression of the human BPI gene and found important functional binding sites for AML1, Sp3, and PU.1, using a combination of *in vitro* experiments as described in the experimental considerations. The identified *cis*-elements are located within 159 bp upstream of the translation start. The human myeloid leukemic cell lines HL60 clone 15, K562, NB4, and U937 were used as experimental models to demonstrate the myeloid-specific activity of the proximal promoter of the human BPI gene. The BPI promoter was inactive in the non-myeloid HeLa cell line. A correlation between promoter activity and endogenous gene expression was found, in as much as that the promoter showed highest activity in HL60 cells, also showing robust expression of endogenous BPI. U937, NB4 and K562 showed lower promoter activity and also a weaker expression of endogenous BPI, as judged by Northern Blot and RT-PCR. By performing transient ectopic expression of the transcription factors C/EBPα or C/EBPε in HeLa cells we also demonstrated not only an induction of promoter activity, but also an induced expression of the endogenous BPI gene, indicating an important role for C/EBPα and C/EBPε in transcriptional regulation of human BPI.

Almost parallel with the investigation of the transcriptional regulation of human BPI gene, we cloned the mouse ortholog to BPI and found that mouse BPI-mRNA is present in testis, epididymis, bone marrow, as well in the mouse Sertoli 15P-1 and the mouse promyelocytic MPRO.C1 cell lines (**Paper II**). However, low expression levels of BPI in mouse bone marrow cells, as compared to the expression levels in human bone marrow cells

(**Paper II**), indicate important differences in the transcriptional regulation between human-and mouse BPI gene. To further investigate this hypothesis, we cloned the promoter region of the mouse BPI gene (**Paper III**). To localize the promoter region of the mouse BPI gene, we used 5' RML-RACE, and found two alternative transcription start sites, which is analogous to what we found in the human BPI gene (**Paper I**). Transient transfection of five different progressive 5' deletion mutants of the mouse BPI promoter into Sertoli 15P-1 and the promyelocytic MPRO.C1 cells showed strongest promoter activity for the 304 bp –and 166 bp long deletion mutants. The shortest deletion mutant of 27 bp showed only 50% of the maximal promoter activity, which indicates a lack of some critical regulatory *cis*-elements, important for the activity of the 166 bp fragment. Based on the result of this indirect luciferase reporter assay, we used the internet-based Transfac program to search for potential transcription factor binding sites in the 166 bp promoter fragment, as we also did in the case of the promoter of the human BPI gene (**Papers I and III**). Potential binding sites for c-myb, USF, NF-κB, MZF-1, GATA-1, AML1, and Sp1/Sp3 were found in the mouse BPI promoter and some of these potential sites were similar to those found in the human BPI promoter.

The lack of potential C/EBP binding sites in the mouse BPI promoter provide a possible explanation for the low expression of BPI mRNA in mouse bone marrow cells, as compared to the high expression levels of BPI mRNA in human bone marrow cells (**Papers I and III**). The human BPI promoter contains a functional and important C/EBP binding site at position –100 upstream of the translation start (**Paper I**). The importance of this C/EBP site is strengthened by the finding that the transcription factors C/EBPε and C/EBPβ bind to the human BPI promoter in ATRA-treated NB4 cells [298], indicating an important role for C/EBP transcription factors in the differentiation-related expression of human BPI in myeloid cells. Since C/EBPε is an ATRA-responsive gene containing a RARE (Retinoic Acid Response Element) within its promoter [43], C/EBPε might be a link between ATRA and human BPI expression. The treatment of the mouse promyelocytic MPRO.C1 cell line with ATRA similarly induced mouse BPI

mRNA expression, and the effect of ATRA was partly dependent of *de novo* protein synthesis. A similar induction of the mouse BPI mRNA was seen in the ATRA-treated Sertoli 15P-1 cells (**Paper II**). These results suggest that also the mouse BPI gene expression is differentiation-related, but, however, no potential RARE, neither potential C/EBP site, was identified in the cloned mouse BPI promoter region (**Paper III**). It is possible that the ATRA-response is mediated by a RARE located in a more upstream regulatory element. The human proximal BPI promoter does also not contain a potential RARE, but here an indirect effect of ATRA was shown by the binding of C/EBPε as already mentioned. Consistent with the absence of RAREs in the proximal promoter of both human and mouse BPI, it was not possible to demonstrate ATRA-responsiveness using the indirect luciferase reporter assay (data not shown).

Transfac database search revealed a potential AML1a binding site at position –32 in the 166 bp mouse BPI promoter , which was not included in the short 27 bp deletion variant. The activity of the 27 bp promoter was significantly reduced, as compared to the larger 166 bp promoter, when transiently transfected in both 15P-1 and MPRO.C1 cells, indicating an important and functional role for this AML-1 site in the transcriptional regulation of the endogenous expression of mouse BPI gene (**Paper III**). Of course, this is not a definitive answer if the mouse BPI promoter has functional binding site for AML-1 or not. First, the Transfac database can give false-positive results or can have missed an potential AML-1 site at another position within the mouse BPI promoter, because this program is based on PWM (position weight matrix). These PWMs are calculated from a set of experimentally defined transcription factor binding sequences and reflect binding specificity for the transcription factor [299]. Secondly, the potential binding site for AML-1 requires further analysis with other methods, including site-directed mutagenesis, and functional binding assays like EMSA or ChIP (as discussed in experimental considerations).

To further compare the mouse and human BPI promoter, we performed a phylogenetic footprinting. Phylogenetic footprinting is the identification of conserved regulatory elements by comparing genomic sequences of ortholog

genes [300]. This computational method is based on the hypothesis that the finding of conserved binding sites when aligning the regulatory sequences of orthologous with eachother, increases the possibility that the binding sites are functional for the transcriptional regulation of the endogenous gene expression.

The gene for mouse BPI is located at chromosome 2 and shows a similar exon-intron organization as the gene for human BPI, which is located at chromosome 20. The mouse BPI gene and human BPI gene encode similar amino acid sequences. Encoded proteins have both antibacterial activity towards gram-negative bacteria and both proteins are, based on these criteria, defined as orthologs to each other (**Paper II**). Given the potential AML1 site in the mouse BPI promoter, it was of interest to determine whether a similar or conserved AML1 site was present in the human BPI promoter. We used the ConSite internet-based program [301] for the alignment of the promoters of human BPI gene and mouse BPI gene. The input promoter sequences we analyzed were the 166 bp long mouse BPI promoter fragment and the 222 bp long human BPI promoter fragment. We found that the potential AML1a site at position –32 in the mouse BPI promoter indeed is conserved in the human BPI promoter at position –142 as shown in **figure 6**. One has to remember that the numeration of the orthologous promoters in paper I and III are not directly comparable. The numeration in the mouse BPI promoter is relative to and upstream of the identified transcription start, while in the human BPI promoter the numeration is relative to the translation start (**Paper I and III**).

The human BPI promoter contains two AML1 sites, one at position –155 and one at position –142 and both are functional as judged by the performed *in vitro* experiments. AML-1 protein bound specifically to the AML1 probe (which contains both sites) as demonstrated by a supershift in EMSA (**Paper I**). The disrupted complex formation when both AML-1 sites are mutated in the unlabeled DNA probe in a competition experiment in EMSA was also in good correlation with the reduced promoter activity when both AML-1 sites are mutated in the 222 bp promoter fragment of human BPI (**Paper I**). Furthermore, deletion analysis of the human BPI promoter

showed reduced promoter activity of the 134 bp fragment as compared with the highest luciferase activity obtained with the 159 bp fragment (**Paper I**). This finding suggest that there is some important cis-element missing in the 134 bp promoter fragment, which is indeed included in the 159 bp fragment. This site is probably the conserved AML-1 site at position –142 and this, together with the results obtained of the EMSA, supports our hypothesis that the conserved AML-1 site is functional in the transcriptional regulation of the mouse BPI gene in the myeloid cell.

The phylogenetic footprinting analysis also indicated a conserved Sp1/Sp3 binding site, which was not found with the Transfac database program when analysing the promoter sequence of mouse BPI gene. This conserved Sp1/Sp3 site in the human BPI promoter is located at position –136 (relative to ATG) and in the mouse BPI promoter it is located at –26 (relative to TS) as shown in **figure 6**. For the mouse BPI promoter, the potential Sp1/Sp3 site at position –15, which was found with Transfac database and not with ConSite database, is like the potential Sp1/Sp3 site at position –26 included within the 27 bp deletion mutant. This latter indicates that both binding sites, respectively, can be responsible for the minimal promoter activity, as demonstrated by the luciferase reporter assays in 15P-1 and MPRO.C1 cells (**Paper III**). The finding that the Sp1/sp3 site at position –26, which is found to be functional in the human BPI promoter (**Paper I**), is conserved, makes the possibility higher that this site also is functional for the transcriptional regulation of the mouse BPI gene. Other conserved sites found by the combination of phylogenetic footprinting and Transfac database search, include USF, Lmo2, another AML-1 site, c-Rel and cap as represented in **figure 6**. If these sites have some functional importance in the myeloid-specific regulation of human-and mouse BPI gene has to be further investigated.

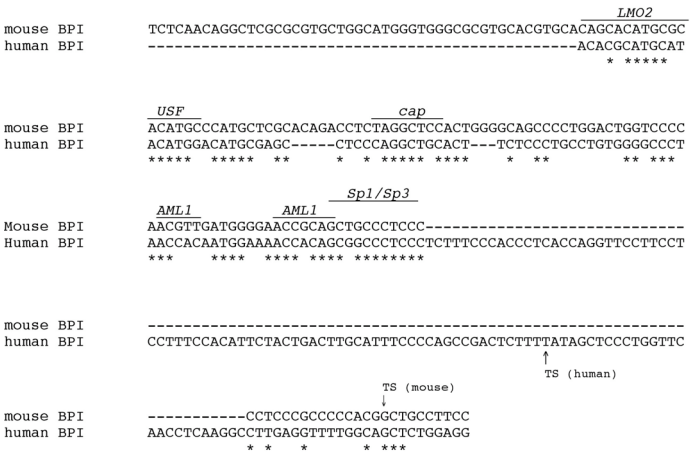

Figure 6. Conserved transcription factor binding sites within the promoters of human - and mouse BPI using phylogenetic footprinting and Transfac database search.

A.2. Is the transcriptional regulation of the BPI genes comparable to other neutrophil granule protein genes? (**Paper I, II,** and **III**)

When we started our investigation of the transcriptional regulation of the human BPI gene in myeloid cells, two observations from the literature made us speculate that human BPI has different transcriptional regulation than other azurophil granule proteins. First, newborn neutrophils are shown to have three-to four-fold less intracellular BPI content than adult neutrophils, while other azurophil granule proteins, α-defensins and myeloperoxidase, are present in identical amounts in neutrophils of newborns and adults [227]. Secondly, the neutrophils of patients with Specific Granule Deficiency (SGD) lack the azurophil granule proteins BPI and α-defensins, while the azurophil granule protein myeloperoxidase is not affected [60]. In the case of SGD, it is therefore suggested that the transcriptional regulation of BPI is similar to that of the specific granule proteins lactoferrin and collagenase, and might be also similar to that of the α-defensins, but differs from transcriptional control of the azurophil granule protein myeloperoxidase. SGD is a very rare disease, only five cases are known worldwide, and some of the SGD patients show homozygous inactivating mutations of the C/EBPε gene, from which is known that the encoded wild-type C/EBPε

protein is important in the regulation of the lactoferrin and collagenase expression [90, 127]. C/EBPε-deficient mice show functional defects of neutrophils very much alike those defects seen in SGD patients. Thus data strongly suggest that C/EBPε is important for the expression of both BPI and α-defensins in myeloid cells. However, at that time Gombart *et al* published its review, indicating that C/EBPε is involved in the molecular basis of SGD, α-defensins and BPI were not known to be present in mouse neutrophils [60, 206], indicating that a comparison between human and mice was difficult to make. Now, we have demonstrated the expression of BPI mRNA in mouse bone marrow cells by using real time RT-PCR and detected BPI protein by immunochemistry (**Paper II**).

Eisenhauer *et al* did not detect α-defensins in more myeloid cells using Northern Blot. However, using more sensitive techniques like real time RT-PCR, it is may be possible to detect low expression levels of α-defensins in mouse neutrophils too.

The lower expression level of BPI mRNA in mouse bone marrow as compared to human bone marrow can be due to lack of a potential C/EBP site as already discussed above, but also the potential 'very' low expression of α-defensins in mouse neutrophils can may be due to this lack of potential C/EBP-site in its promoter.

The human BPI –and α-defensin promoters do contain functional C/EBP sites. C/EBPβ and C/EBPε bind the human BPI promoter *in vivo* [298] and the ectopic overexpression of C/EBPα induces the human BPI promoter in a direct or indirect fashion as it was demonstrated *in vitro* (**Paper I**). The human α-defensin promoters bind C/EBPα and this transcription factor plays an important role in the regulation of α-defensins gene expression in immature myeloid cells [82].

Further, it is demonstrated that BPI and α-defensins are synthesized later than the other azurophil granule proteins, such as MPO (myeloperoxidase), PR3 (proteinase 3) and NE (neutrophil elastase) during the promyelocyte stage of myelopoiesis in the human bone marrow [302, 303]. Both human α-defensins and human BPI, contain potential CDP-sites in their promoters,

which can play a role in the later expression of both proteins during the promyelocyte stage of myelopoiesis, knowing that CDP is a repressor of gene expression, and that the repressor-activity of CDP is downregulated upon differentiation of myeloid cells [82-85, 298]. CDP represses the expression of the human lactoferrin gene but as myeloid cells differentiate beyond the promyelocyte stage, the CDPs repressor-activity is down-regulated which results in the expression of the lactoferrin gene [86, 302]. If the transcriptional regulation of human BPI is similar to that of human lactoferrin, as could be suggested from the observation in SGD patients, CDP could play an important role in the regulation of human BPI gene. However, we have so far not been able to demonstrate a functional role for CDP in the regulation of human BPI and there are no data available from the literature supporting a role for CDP in the expression of α-defensin genes during myelopoiesis. In addition, no conserved CDP site was found when aligning the human and mouse promoters in the phylogenetic footprinting analysis, so the role of CDP in the regulation of both BPI promoters is not clear. CDP is also known to negatively regulate the C/EBPε promoter activity [304], so hypothetically CDP can also indirectly regulate the expression of the human BPI gene via repression of C/EBPε.

Are the transcription factors PU.1, AML1 and Sp3 responsible for the different expression profile of BPI as compared to the other azurophil granule proteins MPO, PR3 and NE?

PU.1 regulates almost every myeloid-specific gene [25], including the human PR3 gene [74] and the MPO gene [70]. The enhancers of both human and mouse MPO contain also functional AML1 sites [45, 64, 72], but it is not demonstrated that AML1 regulates the other azurophil granule proteins like proteinase 3 or the α-defensins. The promoters of human α-defensins contain two potential AML1 sites, which are not found in the PR3 promoter [74, 82]. Therefore, the transcription factors PU.1 and AML1 do not act alone or separately, but most likely bind with other regulatory factors to make the expression profile of human BPI different as compared with the other azurophil granule proteins PR3, NE or MPO.

It is shown for the M-CSF receptor, for example, that its promoter contains three adjacent and functional binding sites for C/EBPα, AML1, and PU.1 and that AML1 physically interact with PU.1 and C/EBPα to activate the M-CSF receptor promoter in a synergistic fashion [136]. However, we could not find a direct binding of C/EBPα to the human BPI promoter [298], but ectopic expression of C/EBPα in HeLa cells was able to strongly activate the human BPI promoter as compared to the ectopic expression of C/EBPε. We can only speculate that the ectopic expression of C/EBPα is able to induce the expression of other transcription factors or can interact with other factors, such as Sp1/Sp3, which are endogenously expressed in HeLa cells [305, 306], and in this way induce the human BPI promoter. Besides direct binding of AML1 and PU.1, we found that Sp3 also binds specifically to the human BPI promoter, and hereby, we demonstrate that the human BPI gene is the first myeloid-specific gene that is positively regulated by a direct and functional binding of Sp3.

Sp3 belongs, together with Sp1, to the Sp family of zinc finger DNA-binding proteins and shows more than 90% identity to Sp1 in the zinc finger DNA-binding region [142, 143]. Sp1 and Sp3 are both ubiquitously expressed, and they compete for binding to GC boxes in several cell types [142]. Sp3 can not only work as an activator, but can also work as an repressor [307]. Recently, it was shown that Sp3 is a target for sumoylation which is essential for its repressive activity [154]. We can only speculate on that during early myelopoiesis, the sumoylated form of Sp3 represses the BPI expression and later it can in its unsumoylated form activate the BPI gene expression.

B. A mouse antibacterial ortholog to human BPI (**Paper II**)

There are numerous of genes that have been conserved through evolution and their protein products can be found to be expressed in many different species, like human and mice. Genes or proteins from different species that share a common ancestor and have the similar functions, are defined to be

orthologs. In **paper II**, we identified a mouse ortholog to human BPI, which was similar in structure and function and, like human BPI, is found to be expressed in testis and in bone marrow cells.

We cloned a mouse BPI cDNA with a predicted molecular weight of 53 kDa and its predicted amino acid sequence was 53% identical and 71% similar to the amino acid sequence of human BPI. The identical or conserved amino acids, include the cysteines at position 132, 135, and 175 (as shown in figure 1 in paper II) and which are like in the human BPI sequence, located in the N-terminal half of the protein [217]. The cysteine residues at position 135 and 175 were previously demonstrated to play essential roles in the stability and function of the human BPI protein [308, 309], and are also shown to be conserved in the other related proteins, including LBP, CETP and PLTP, but not the cysteine residue at position 132 [309].

The three-dimensional (3D) - structure of the human BPI protein shows an unusual 'boomerang' structure (as shown in figure 2 in Paper II) formed by two 'quite' similar domains, as it was determined by Beamer *et al [214].* Each domain contains a 'barrel' formed by two α-helices and a highly twisted antiparallel β-sheet. In the center of the protein, the two barrels are connected by a smaller antiparallel β-sheet, which consist of a proline rich sequence [214, 238, 310], which is also conserved between human BPI and the predicted mouse BPI (as shown with a line in figure 1 in paper II). A disulphide bridge that anchors the β-sheet to one of the α-helices in the N-terminal domain of the human BPI protein is formed by the two conserved cysteines at position 135 and 175 and are suggested to play also a role in the structure/stability and eventually similar function of the cloned mouse BPI. Based on the knowledge that the N-terminal domain is responsible for the bactericidal, LPS-binding, and LPS-neutralizing activity of human BPI [311, 312] and the conserved cysteine residues, which play a role in this biological activity [308, 309], we hypothesized that the cloned mouse BPI has similar functions as the human BPI protein. Therefore, we stably expressed recombinant human- or mouse proteins in HEK293 cells. These cells have no cytoplasmic organelles and we supposed that they should secrete their products into the medium. This conditioned medium was harvested and

analyzed for antibacterial activity toward a gram-negative bacteria, the *E. coli* strain J5 [313]. We found that the antibacterial activity against *E.coli* strain J5 of the recombinant mouse BPI was similar to that of recombinant human BPI, suggesting that mouse BPI has similar function against gram-negative bacteria as human BPI. The intact gram-negative bacteria, *E. coli* strain J5, as used in this *in vitro* assay, is a strain bearing short-chain lipolysaccharide (LPS) in its outer membrane and is defined as a rough strain of *E. coli*. It is previously demonstrated that rough strains of *E.coli* are more sensitive to BPI than smooth strains of *E.coli* that produce LPS with varied chain lengths [314]. These smooth strains or also called wild-type strains, and are shown to modify the average length of the polysaccharide chain in response to changes in growth medium [315]. Therefore, although the use of the rough strain *E. coli* J5 in our *in vitro* bacterial killing assay gives us an answer whether mouse BPI show antibacterial activity. It does not tell whether the antibacterial activity is equally effective against other bacteria strains.

The polyclonal antibodies against our cloned mouse BPI, which were succesfully used in the immunocytochemistry assay, did not work in assays like Western blot or in our bacterial killing assay. For this reason, it was impossible to analyze the expression/synthesis of endogenous mouse BPI at the protein level in mouse cell lines of interest. On the other hand, the mouse promyelocytic MPRO.C1 cell line, the mouse Sertoli TM4- and 15P-1 cell lines were analysed by real time RT-PCR and were found to express endogenous mouse BPI mRNA.

Based on the finding that mRNA of mouse BPI and human BPI was not only detected in bone marrow and promyelocytic cell lines, but also in tissues like testis and epididymis, we were interested in other antimicrobial proteins and peptides found in the tissues of the male reproductive organ. Indeed, one study demonstrate the existence of a number of mRNA transcripts of β-defensins with specific expression in distinct regions of the mouse epididymis, suggesting that these proteins play a role either in host defense or in other functions in this organ. The epididymis is a tubular male reproductive structure that lies on and around each testicle, connecting it

with the vas deferens [316] and is known to play an important role in the maturation of sperm, which after their production in testis, pass through the epididymis [317]. Another study showed that the epididymis-specific β-defensin, which has an ortholog in humans [318], have antimicrobial activity similar to that of other β-defensins [319], but that β-defensins also play also a role in the sperm maturation [320]. Human epididymis also seems to have high protein levels of hCAP-18 [321], known as the only human cathelicidin, first isolated in the specific granules of neutrophils [322]. The hCAP-18 is attached to the spermatozoa and is suggested to play a role in antimicrobial defense during human reproduction [323]. An immunocytochemistry assay indicated that a protein, called SOB3, is located in the acrosome of human spermatozoa and seems to play a role when the spermatozoa will pass through the zona pellucida during the fertilization of the oocyte [324]. Based on the alternative functions of these proteins found in the male reproductive organ, we can speculate that mouse - and human BPI might also have other functions than antimicrobial activity.

C. *The Single Nucleotide Polymorphism (SNP) in the proteinase 3 promoter* (**Paper IV**)

Genetic variation or polymorphisms with functional consequences can occur in the coding DNA (exonic sequence) or in noncoding, regulatory DNA (promoter-or enhancer sequence). Functional polymorphisms in the coding sequence of a gene gives rise to changes in amino acid sequence of the encoded protein, while functional poylmorphisms in the regulatory sequences can change the mRNA levels. Most genetic variations exist as a single base change in the DNA sequence, known as Single Nucleotide Polymorphism (SNP), and this ocurs approximately once every 1000 nucleotides [325]. Sometimes SNPs are associated with human diseases. We hypothesized that the –564 A/G SNP in the promoter region of proteinase 3, which introduces a new potential Sp1 binding site, may be responsible for the observed up-regulated proteinase 3 membrane expression in myeloid cell

of patients with Wegener's Granulomatosis (WG). Our hypothesis was based on the finding that the –564 A/G SNP was over-represented in patients with WG as compared to healthy controls [326]. Similarly, a common polymorphism (-463 G/A) in the myeloperoxidase (MPO) promoter, which creates an additional Sp1 site, resulted in a 25-fold increase of transcriptional activity [327] and this SNP might be associated with WG [328], or not [329]. To test this hypothesis, we transiently transfected promyelocytic HL60 and NB4 cells, monocytic U937 cells, and cervix carcinoma HeLa cells with three variants of the proteinase 3 promoter (-564 A/G; -621 A/G; and wild-type), as represented in figure 1 in **paper IV**, and analyzed promoter activity. We found highest promoter activity in HL60 cells, which also was the cell line showing highest levels of endogenous gene expression. No significant promoter activity was seen in the HeLa cells, demonstrating that the analyzed proteinase 3 promoter shows a myeloid-specific activity. However, within each cell line, our data fail to reveal any significant differences in promoter activity among the three variants, thus strongly arguing against our hypothesis. A possible explanation to the absence of any effect on transcription from the new Sp1 site is that the potential Sp1 site at position -564 might be too far separated from the TATA-box to give an induction of the proteinase 3 promoter activity.

In the case of the polymorphism of the MPO promoter, as mentioned above, we speculate that the additional Sp1 site is functional, because it is located at close distance to the alternative transcription start site P2 in this MPO promoter [330]. Although it is shown in the study of Lin *et al* that the wild-type MPO gene is transcribed *in vivo* primarily using the P1 promoter and that low levels of transcription occurs at the alternative P2 site, we can not exclude that the additional Sp1 site near this P2 site in the mutant MPO promoter is able to increase the level of transcription. A previous study showed a functional and important Sp1 site near the P1 transcripion start site in the wild-type MPO promoter, indicating again that close distance of Sp1 sites to the transcription start site is essential for increase of promoter activity [63].

In conclusion, we demonstrated that the –564 A/G SNP in the proteinase 3 promoter is not the answer why resting neutrophils or monocytes of WG patients have increased proteinase 3 expression.

V. Conclusions

- The BPI mRNA expression during myelopoiesis is regulated by the transcription factors AML-1, PU.1 and Sp3. The reason that BPI is later expressed than other azurophil granule proteins, like myeloperoxidase (MPO), proteinase 3 (Pr3) and neutrophil elastase (NE) is still unknown, but we can speculate, based on our results in this study, that the transcription factor Sp3 can play an important role in the unique expression profile of BPI during myelopoiesis. BPI is the first myeloid-specific gene shown to be regulated by Sp3.

- Human - and mouse BPI are structurally and functionally conserved and are defined to be orthologs to each other. Mouse BPI mRNA was detected in testis, epididymis, bone marrow cells and Sertoli-and promyelocytic cell lines. Mouse BPI mRNA expression was induced when Sertoli cells 15P-1 and the promyelocytic MPRO.C1 cells were stimulated with ATRA. In contrast to human BPI, the ATRA- induction of mouse BPI mRNA expression seems, at least partly, to be independent of *de novo* protein synthesis, indicating direct effects of ATRA on the mouse BPI promoter.

- Mouse bone marrow cells express much lower levels of BPI mRNA as compared to human bone marrow cells. We found that the proximal mouse BPI promoter lack a potential C/EBP binding site, which can be

responsible for the lower expression of BPI mRNA in mouse bone marrow cells. Furthermore, we found a conserved, but potential AML-1 binding site in the promoters of human -and mouse BPI gene. However, further studies are necessary to demonstrate the functionality of the AML-1 site.

- The –564 A/G Single Nucleotide Polymorphism (SNP), which introduces an potential Sp1 binding site in the promoter of proteinase 3 gene, is not responsible for the increased expression of proteinase 3 in myeloid cells of patients with Wegener's Granulomatosis. Further investigations must be done to understand the molecular mechanisms behind the increased proteinase 3 expression in myeloid cells or other risk factors in the pathogenesis of Wegener's Granulomatosis.

Populärvetenskaplig sammanfattning på Svenska
(Samenvatting in het Zweeds)

Våra vita blodceller bildas i benmärgen och när de mognat färdigt hamnar de i blodcirkulationen. Det finns olika typer av vita blodceller och varje typ har sin egen funktion. En speciell typ av vit blodcell, nämligen den myeloida vita blodcellen (neutrofilen), spelar en mycket viktig roll i kroppens första försvar mot invaderande bakterier. När bakterier kommer in i kroppen någonstans, lämnar de myeloida vita blodcellerna blodcirkulationen och vandrar till det stället och dödar de invaderande bakterierna. Avdödning av bakterier sker med hjälp av bakteriedödande proteiner som cellerna själva producerar och förvarar i små behållare, sk granula, inuti i cellerna. Livslängden för mogna neutrofiler är begränsad till några dagar och behöver därför hela tiden ersättas av nya celler från benmärgen. Mognadsprocessen av neutrofiler i benmärgen sker i ett antal på varandra följande steg, varvid olika sorters bakteriedödande proteiner bildas och lagras i granula. Beroende på när proteinerna bildas under mognadsprocessen, hamnar de i olika sorter granula i neutrofilen.

Proteiner bildas med hjälp av ärftlig information som finns i våra gener. Gener är uppbyggda av DNA och finns i cellens kärna, vilken är avgränsad från resterande delen av cellen, cytoplasman. När en gen uttrycks betyder det att den läses av på ett sådant sätt att den blir översatt till det protein genen innehåller information om. Instruktion om vilka proteiner som ska tillverkas, i vilka mängder och på vilka tidpunkter under utmognaden, är alltså förvarad i våra gener.

När en gen uttrycks bildas först en slags kopia av den. Denna kopia, som kallas mRNA, transporteras ut ur kärnan till cytoplasman där den fungerar som mall för de proteiner som skall tillverkas. Bildandet av mRNA styrs med hjälp av så kallade transkriptionsfaktorer. Transkriptionsfaktorer är proteiner som binder till vissa delar av reglerande DNA i gener och kan därmed bestämma om genen ifråga skall uttryckas eller inte. Beroende på vilka, och på vilket sätt, transkriptionsfaktorer binder till DNA, bildas antingen många eller få mRNA-kopior, eller ibland inga mRNA-kopior alls. Exakt hur och när transkriptionsfaktorer binder till DNA för att skapa mRNA-kopior är en

komplex process och mycket forskning pågår för att förstå detta helt. I min avhandling har jag undersökt hur och när mRNA-kopior av vissa gener bildas under mognadsprocessen av neutrofiler.

I **studie I** har vi identifierat några transkriptionsfaktorer som spelar en viktig roll i bildningen av mRNA-kopior som leder till produktionen av det bakteridödande proteinet ”bactericidal permeability increasing protein” (BPI) under mognadsprocessen av neutrofiler. Resultaten har gett oss ökad insikt i och förståelse för varför BPI inte bildas vid vissa sjukdomar.

I **studie II** har vi upptäckt att BPI finns inte bara i myeloida vita blodceller hos människa, utan att också i blodceller från mus bildas små mängder av BPI-proteinet. BPI hos mus är inte tidigare upptäckt av andra forskare. Vi fann också att mRNA-kopior av BPI bildas i vissa celler av manliga könsorgan i både människa och mus.

I **studie III** var jag nyfiken på varför det bildas mycket mindre mRNA-kopior av BPI genen i benmärgen hos mus, i jämförelse med människa. Därför ville jag undersöka om vissa transkriptionsfaktorer som spelar en viktig roll i uttrycket av BPI genen hos människa är viktiga också för uttrycket av BPI-mRNA hos mus. Tyvärr har jag inte hunnit ända fram i detta delprojekt, men mina resultat antyder ändå att BPI-genen i mus saknar de inbindningsställen för en viss typ av transkriptionsfaktor. Denna transkriptionsfaktor är viktig för bildningen av stora mängder av BPI proteinet i människans vita blodceller och eftersom motsvarande faktor hos musen inte kan verka på BPI-genen där, skulle det kunna förklara varför BPI-nivåerna är låga i myeloida celler hos mus.

I **studie IV** undersökte jag hur det reglerande DNA i en annan gen, nämligen genen som kodar för proteinas 3, är uppbyggd för att bättre kunna förstå varför myeloida vita blodceller av patienter med en sjukdom som kallas Wegener’s Granulomatosis (WG) bildar större mängder av proteinas 3-proteinet, i jämförelse med friska personer. Överproduktionen av proteinas 3 kan kanske vara en bidragande orsak till att dessa patienter utvecklar farliga inflammationer i små blodkärl. Många patienter med WG har en särskild variant av det reglerande DNA i proteinas 3-genen. Jag hypotetiserade att denna variant orsakar att fel transkriptionsfaktor binder in, vilket i sin tur leder till produktion av stora mängder proteinas 3-protein. Men min hypotes visade sig vara fel. Mer

forskning krävs alltså för att undersöka orsaken till överproduktionen av proteinas 3 i WG patienter.

Samenvatting in het Vlaams

(Populärvetenskaplig sammanfattning på flamländska)

Onze witte bloedcellen worden gevormd in het beenmerg en wanneer ze rijp zijn komen ze in de bloedcirculatie terrecht. Er zijn verschillende soorten witte bloedcellen, die elk hun eigen functie uitoefenen in het immuunsysteem van ons lichaam. Een bepaalde soort witte bloedcel, namelijk de myeloïde witte bloedcel (de neutrofiel), speelt een belangrijke rol in de eerste verdediging tegen binnengedrongen bacteriën. Tijdens een ontstekingsreactie, kan de myeloïde witte bloedcel de bloedcirculatie verlaten door de wand van de allerkleinste bloedvaten om in de ontstoken weefsels de binnengedrongen bacteriën te vernietigen. Het vernietigen van bacteriën gebeurt met de hulp van bacteriedodende eiwitten die geproduceerd worden door de cel zelf en die als het ware bewaard worden in blaasjes (granules) binnenin de neutrofiel. De levensduur van de rijpe neutrofiel is beperkt tot enkele dagen en vandaar dat ze voortdurend moet vervangen worden door nieuwe cellen vanuit het beenmerg. Het rijpingsproces van de neutrofielen in het beenmerg verloopt in een aantal opeenvolgende herkenbare stappen, waarbij verschillende soorten bacteriedodende eiwitten worden aangemaakt die bewaard worden in de granules. Afhankelijk van het tijdstip wanneer de eiwitten worden aangemaakt tijdens het rijpingsproces komen ze terecht in verschillende soorten granules binnenin de neutrofiel.

Eiwitten worden aangemaakt aan de hand van erfelijke informatie die vastligt in onze genen. Onze genen zijn opgebouwd uit DNA en bevinden zich in de kern van de cel, afgezonderd van het grootste resterende deel van de cel, het cytoplasma. Wanneer een gen tot expressie komt wil dit zeggen dat een gen wordt afgelezen zodanig dat het kan vertaald worden tot de aanmaak van

eiwitten waarvan het gen informatie bevat. De instructies hoe eiwitten worden aangemaakt, in welke hoeveelheden en op welke tijdstippen van het rijpingsproces ligt allemaal vast in onze genen. Wanneer een gen tot expressie komt, wordt er eerst van het DNA een soort kopie gemaakt. Deze kopie, dat men mRNA noemt, wordt dan getransporteerd van de celkern naar het cytoplasma waar de eiwitten daadwerkelijk worden aangemaakt met de hulp van de instructies die deze mRNA-kopie bevat.

Het maken van mRNA-kopies gebeurt met de hulp van zogenaamde transcriptiefactoren. Transcriptiefactoren zijn eiwitten die zich met bepaalde delen van het regulerende DNA binden en op deze manier beslissen of het gen in kwestie to expressie zal komen of niet. Afhankelijk van de manier waarop en welke transcriptiefactoren het regulerende DNA binden, worden er veel of weinig mRNA-kopies aangemaakt, of soms worden er zelfs geen mRNA-kopies aangemaakt. Het hele proces van hoe en wanneer transcriptiefactoren binden met het regulerende DNA om mRNA-kopies aan te maken is ingewikkeld en er worden heel veel studies gedaan om dit proces beter te begrijpen.

In mijn thesis heb ik me vooral geconcentreerd op hoe en wanneer mRNA-kopies van bepaalde genen worden aangemaakt tijdens het rijpingsproces van de neutrofiel.

In **studie I**, hebben we een aantal transcriptiefactoren geïdentifieerd die verantwoordelijk zijn voor het aanmaken van RNA-kopies die in hun beurt tot de aanmaak van het bacteriedodende eiwit 'Bactericidal/Permeability-Increasing protein' (BPI) leiden tijdens het rijpingsproces van de neutrofiel. De identificatie van deze transcriptiefactoren kan ons meer inzicht geven waarom in bepaalde ziektes het eiwit BPI niet wordt aangemaakt.

In **studie II**, ontdekten we dat het eiwit BPI niet alleen wordt aangemaakt in de myeloïde witte bloedcellen van de mens, maar dat zelfs muizen het eiwit BPI in kleinere hoeveelheden aanmaken in hun myeloïde witte bloedcellen. BPI in muizen was voorheen nog nooit aangetoond door andere wetenschappers. We zagen ook dat mRNA-kopies van het BPI gen worden aangemaakt in bepaalde cellen van het mannelijk geslachtsorgaan van zowel muis als mens.

In **studie III**, was ik nieuwsgierig waarom dat de myeloïde witte bloedcellen van de muis veel minder mRNA-kopies van het BPI gen aanmaken in vergelijking tot de mens. Daarom wilde ik nagaan of de transcriptiefactoren die verantwoordelijk zijn voor de aanmaak van mRNA-kopies van het BPI gen in de cellen van de mens ook belangrijk zijn in de cellen van de muis. Helaas was de tijd tekort om deze studie af te maken, maar mijn resultaten wijzen erop dat het BPI gen in muizen een bindingplaats voor een bepaald type van transcriptiefactor mist. Deze transcriptiefactor is belangrijk voor de aanmaak van grote hoeveelheden van het BPI eiwit in de myeloïde witte bloedcellen van de mens en omdat de muis dat bepaald type van transcriptiefactor mist om BPI mRNA-kopies aan te maken, kan dit een mogelijke verklaring zijn waarom BPI in veel kleinere hoeveelheden wordt aangemaakt in de muis.

In **studie IV**, bestudeerde ik hoe het regulerende DNA van een ander gen, namelijk het gen dat de informatie bevat om het eiwit 'proteinase 3' aan te maken, is opgebouwd om te kunnen verstaan waarom de myeloïde witte bloedcellen van patiënten met de ziekte Wegener's Granulomatosis (WG) grotere hoeveelheden van dit eiwit proteinase 3 aanmaken in vergelijking tot gezonde mensen. Deze overproductie van het eiwit proteinase 3 is misschien de oorzaak dat deze patiënten ernstige ontstekingen krijgen in de allerkleinste bloedvaten. Vele patiënten met WG hebben een speciale opbouw van het regulerende DNA in het proteinase 3 gen. Ik ging er vanuit dat deze speciale opbouw van het regulerende DNA ervoor zorgde dat een verkeerde transcriptiefactor kan binden met dit DNA en er dus zo zou voor kunnen zorgen dat er te grote hoeveelheden van dit proteinase 3 eiwit worden aangemaakt. Ik kwam tot de waarneming dat dit niet zo bleek te zijn. Dus er moet meer onderzoek worden verricht om de oorzaak van deze overproductie van het eiwit proteinase 3 in WG patiënten beter te begrijpen.

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