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Characterization Of The Human Neutrophil Elastase Promoter.

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CHARACTERIZATION OF THE HUMAN NEUTROPHIL ELASTASE PROMOTER

by

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A DISSERTATION

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ABSTRACT OF DISSERTATION
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Title Characterization of the Human Neutrophil Elastase Promoter.

The Human Neutrophil Elastase (HNE) Is One of the Many Genes Whose Expression Is Developmentally Regulated During Myeloid Differentiation. The Expression of HNE Is Restricted To the Late Stages of Promyelocyte Differentiation In the Normal Bone Marrow. The Exact Mechanisms That Govern the Expression of Myeloid-specific Expression of Lineage Restricted Genes Have Not Been Fully Discerned. This Dissertation Is Aimed At Elucidating the Mechanisms of Regulation of Expression of the HNE Gene. We Show That A 30 Bp Element, Located Between -76 And -106 In the 5' Flanking Region, Is Responsible For Directing Myeloid-specific Expression of the HNE Promoter. This Element Binds Several Proteins Present In the Nuclear Extracts of Myeloid Cells. We Show That the Main Factor Responsible For the Functional Activity of the HNE Promoter Is the Transcription Factor, PU.1. This Factor, When Expressed In Non-myeloid HeLa Cells, Directs Expression From the HNE Promoter In Non-myeloid Cells. The Level Of PU.1 Protein Did Not Significantly Alter Upon Differentiation Of Myeloid Cells, And Hence The Promoter Was Not Stage-restricted In Expression. Additional Sequences Appear To Be Necessary For The Stage-restricted Expression of HNE, And Our Further Studies Are Aimed At Determining These Mechanisms. This Is the First Granulocyte-specific Gene Shown To Be Regulated By PU.1 And May Provide Useful Insights Into the Mechanisms of Regulation of Other Myeloid Genes.

Abstract Approved by: Committee Chairman T. A. Rado

Program Director Susan Jackson

Date 4/6/95 Dean of Graduate School Joel Glasser

DEDICATION

This dissertation work is dedicated to my parents, Sitha Sampathkumar and S. Sampathkumar, for their support and understanding throughout my graduate studies.

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PU.1 REGULATES THE EXPRESSION OF HUMAN NEUTROPHIL ELASTASE PROMOTER

INTRODUCTION

This dissertation consists of three chapters formed by papers that have been submitted or accepted for publication in peer reviewed journals. The first chapter consists of a review of the recent advances that have occurred over the past three years in the field of myeloid specific gene expression. This review summarizes the major myeloid specific promoters that have been studied so far and gives a detailed description of the transcription factors that direct myeloid specific gene expression. This introductory review helps to place in perspective the body of work regarding the expression of Human Neutrophil Elastase (HNE), which forms the dissertation.

The second paper, published in the Journal of Biological Chemistry volume 269 pages 32626-32633, identifies the main regulatory elements that are present in the promoter of the Human Neutrophil Elastase gene. A 30 bp element, conserved among several myeloid specific promoters, was identified as the main cis acting regulatory sequence within the first 150 bp of the transcriptional start site of the HNE gene. No other regulatory elements within the first 1000 bp of the HNE promoter were found to contribute significantly to transcriptional activity in myeloid cells. The deletion of the 30 bp element from the HNE promoter results in abolition of myeloid specific expression of the HNE promoter. This 30 bp element was shown to bind several proteins found only in the nuclear extracts of myeloid cells.

The third paper, submitted to the journal, Molecular and Cellular Biology, identifies the factors that bind the 30 bp element. It was shown that PU.1, a B cell and macrophage specific transcription factor, binds to the PU.1 binding site within the 30 bp element. Multiple phosphorylated forms of PU.1 were shown to exist in the nuclear extracts of myeloid cells, and protein-protein interactions were shown to occur between

PU.1 and other proteins present in the nuclear extracts of myeloid cells. PU.1, expressed in non-myeloid HeLa cells, transactivated expression from the HNE promoter in HeLa cells.

REGULATORY FACTORS INVOLVED IN MYELOID-SPECIFIC GENE EXPRESSION

by

SAMPATHKUMAR SRIKANTH AND THOMAS A. RADO

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INTRODUCTION

Normal hematopoiesis involves a series of coordinated molecular events resulting in the progressive proliferation and differentiation of committed stem cells into the different hematopoietic lineages. Myelopoiesis is one such coordinated event leading to the formation of mature granulocytes and macrophages from the committed GM-CFU. This process involves the spatial and temporally coordinated developmental regulation of different genes specific to various stages in the maturation of granulocytes and macrophages. Thus, the phenotype of the cells at different stages of myelopoietic maturation is the net result of the various genes that are expressed at each stage of development. Lack of coordinated expression or aberrant expression of myeloid specific genes results in abnormal phenotypes and may result in malignant transformation or lineage infidelity of the cells involved. To aid the molecular understanding of the events occurring at various stages of myeloid differentiation, various genes that are specifically expressed only in myeloid cells were cloned and analyzed. Lubbert et al. reviewed more than 40 different human myeloid specific genes that have been identified and the myeloid leukemic cell lines that were used to study the developmental regulation of these genes (1). Since then, the mechanisms of regulation of expression of several human and mouse myeloid genes have been studied, and the factors responsible for the regulated expression of these genes have been characterized. This review is aimed at summarizing the recent advances in the study of human myeloid gene expression and the transcription factors that regulate the expression of these genes.

Several groups attempting to understand the mechanisms that regulate the expression of myeloid specific genes searched for sequences that are common motifs in the promoters of these genes (2-4). Several elements that are conserved among the different myeloid specific genes were identified and were proposed to have functional role in the transcriptional regulation of these genes. It was expected that myeloid specific regulation be mediated by novel myeloid specific transcription factors that bound

conserved elements in the promoters of these genes. Other tissue specific transcription factors like the GATA and the MyoD family of proteins, which were restricted in expression to erythroid and muscle tissues, respectively, and the role of these factors in the tissue specific regulation of genes expressed in these tissues have been well characterized (5-8). Contrary to expectations, no such myeloid specific transcription factors have been reported so far, however, some myeloid specific transcription factors like the zinc finger transcription factor MZF-1 have been reported (9). The role of this factor in the regulation of myeloid specific factors has not been characterized so far. In contrast, the transcription factors described so far to play a role in the transcription of myeloid specific genes have tissue distribution not restricted only to the myeloid lineages. The most prominent example of this is PU.1, an Ets-related proto-oncogene, expressed in most hematopoietic cells except T cells (10-12). Other factors like Sp1, present in most tissues, may act combinatorially with tissue specific factors to regulate expression of genes (13). So far, no other transcription factor has been directly implicated in the expression of myeloid specific genes.

Other global regulatory mechanisms that occur specifically in myeloid cells have been observed, although the role of these mechanisms in the regulation of genes is yet to be demonstrated. These mechanisms include tissue and stage specific variations in developmental specific DNAse I hypersensitive sites in the flanking regions of the myeloperoxidase genes (14-16). In this context, it is interesting to note that at least two different clusters of serine proteases that are expressed at the promyelocytic stages of granulocytopoiesis have been reported. These are the Elastase group of serine proteases located within 35 kb of DNA on the p terminus of chromosome 19 and the cathepsin G group of proteases located on chromosome 14q11 (17, 18). The role of regulatory mechanisms like locus control regions and tissue specific hypersensitive sites, which direct stage specific domain opening, can be envisaged. Such mechanisms control the

developmental switching at the β-globin locus that occurs during development, and these mechanisms have been well characterized (19-24).

Another mechanism by which global regulation is accomplished is by dynamic regulation of DNA methylation. Correlation between DNA methylation and differentiation has been well characterized (25-27). The methylation of the CpG islands down-regulates transcription in most genes by several mechanisms including regulation of binding of Sp1 protein to its recognition site (28, 29). This also allows the synchronization of the cell cycle events and transcription because methylation only occurs when DNA is replicated. Differential methylation of the sequences in the 5' flanking region has been reported in a few myeloid specific genes including myeloperoxidase and mouse lysozyme (14, 30, 31). It is conceivable that these global regulatory mechanisms may provide a mechanism of overall orchestration of gene regulation, which can be regulated based on the milieu of the myelopoietic cells.

Although the role of these mechanisms in the regulation of some of the myeloid specific genes has been indirectly suggested, the main common theme in the regulation of all the myeloid specific genes whose regulation has been studied so far is the regulation by the transcription factor, PU.1. We shall examine in greater detail the role of this transcription factor in myeloid specific gene expression.

The role of transcription factor, PU.1, in myeloid specific gene expression .
CD11b promoter .

The first human myeloid specific promoter described to be regulated by PU.1 is the promoter for the CD11b gene (32-35). This gene encodes for the α1 integrin component of the Mo-1 cell surface marker found on the surface of granulocytes and macrophages. The expression of protein is limited to the mature stages of myelopoiesis. The promoter does not contain a TATA nor a CCAAT element, which is a characteristic of many myeloid genes expressed at more mature stages of development. Instead, there are at least two different PU.1/Ets motifs within the first 150 bp element. Pahl et al.

identified that a PU.1 binding site 5' TTCTCCTT 3' located between -21 to -14 of the CD11b promoter binds PU.1 and directs the myeloid specific expression of the CD11b promoter (33). This sequence for the binding site of PU.1 varies considerably from the consensus site for PU.1 binding 5' GAGGAA 3'. Such a consensus binding site is found at position -140 of the CD11b promoter but does not bind PU.1 nor contribute to functional activity (33). The sequences surrounding PU.1 binding site have been shown to be critical for PU.1 recognition of its cognate site, and this allows PU.1 to bind to sequences that vary considerably from the consensus binding sequence (33, 36).

Additional deletional and mutational studies on the first 92 bp of the CD11b promoter showed that the a Sp1 site in the first 80 bp of the transcriptional site binds to Sp1 and functionally regulates transcription form the CD11b promoter (34). It was noted that although *in vitro* studies showed that Sp1 protein bound to this site in both myeloid U 937 and non-myeloid HeLa cells, *in vivo* footprinting studies showed that Sp1 bound to the CD11b promoter only in U 937 cells (34). Sp1 is glycosylated and phosphorylated at different residues thereby allowing for differential regulation (37,38). The Sp1 site contains a CpG site that is the target for cellular methylases, and the Sp1 protein binding to this site may be mediated by differential methylation. Thus, the *in vivo* regulation of Sp1 binding to its cognate site can be regulated but the exact mechanism by which this occurs on the CD11b promoter is still unclear (34).

CD18 promoter .

The promoter for human CD18 (β chain of the integrin adhesion protein) has been studied (39). Bottinger et al. reported that the main regulatory elements within the promoter consist of two inverted Ets elements (39). They show that the region between -302 and +19 was sufficient for cell restricted and phorbol-ester inducible expression of the CD18 promoter. The functional elements consist of closely spaced inverted repeats at -81 to -68 sequence 5' GAGGAA 3' (Box A) and -55 to -41 sequence 5' CAGGAA 3' (Box B) and these elements bound several complexes present in myeloid cells. They also

show that PU.1 binds to the Box B and GABP related proteins bind to the Box A of the promoter, and these elements interact to confer the tissue and stage restricted expression to the CD18 promoter.

Macrophage-colony stimulating factor- receptor (M-CSFR) promoter .

The promoter for M-CSFR was characterized and consists of two distinctly different promoters that direct the expression of this gene in macrophages and in trophoblasts (40). The murine promoter, responsible for tissue specific expression, was first identified and reported to consist of multiple PU.1/Ets motifs. This promoter was found to function as a promiscuous promoter, and lineage restricted transcriptional elongation allowed selective expression only in macrophages (41,42). The promoter for the human M-CSFR gene was characterized and consists of an PU.1/Ets binding motif 5' GGGGAA 3' (-33 to -51) just upstream of the transcriptional start site (43). PU.1 specifically bound to the sequence and activated tissue specific transcription. PU.1, cotransfected with promoter constructs into HeLa cells, cotransactivated the expression from the wild type M-CSFR promoter but not from the promoter with a mutated PU.1 binding site (43).

Neutrophil elastase promoter .

The promoter for the Human Neutrophil Elastase gene has been recently characterized (44-46). The main promoter consists of the PU.1/Ets motif 5' GAGGAA 3' located between -82 and -88 of the 5' flanking region (45). The sequences within the first 100 bp from the transcriptional start site confer myeloid specificity to the HNE promoter. It was clearly shown that PU.1 binds to this sequence in the promoter and activates transcription. PU.1, when cotransfected with HNE promoter constructs into HeLa cells, transactivated expression from the HNE promoter. The mutation of the PU.1 binding site affected cotransactivation by PU.1 in HeLa cells (46).

Recently, the Murine Neutrophil Elastase promoter has been characterized and found to contain several regions of conserved sequences to the Human Neutrophil

Elastase promoter. Similar to the human promoter, the main elements responsible for transcriptional activation lie within the first 100 bp of the transcriptional start site (47). PEBP2/CBF, the murine homolog of the AML-1 gene, binds to the consensus binding site 5' GGCCACA 3' located within the first 80 bp of the 5' flanking region resulting in the transcriptional activation of the murine promoter. Additional PU.1, c-myb and c/EBP binding sites were shown to contribute to transcriptional activity (47).

TABLE 1

LIST OF KNOWN PU.1 BINDING SITES IN THE PROMOTERS AND ENHANCERS OF HEMATOPOIETIC SPECIFIC GENES

Human Neutrophil Elastase	5' GAGGAA 3'
CD11b	5' GGAGAA 3'
CD18	5' GAGGAA 3'
CD11a	5' GAGGAA 3'
MCSFR	5' GGGGAA 3'
FCγR	5' AGAAAAG 3'
Ig κ 3' enhancer	5' GAGGAA 3'
Ig λ 2-4 enhancer	5' AAGGAA 3'
Ig J chain promoter	5' GCAGAA 3'
Ig μ heavy chain enhancer	5' GGGGAA 3'
Intervening sequence-2 of β-globin	5' AGGGGAA 3'
SV-40 enhancer	5' GAGGAA 3'
IL-4 intronic enhancer	5' CAGGAA 3'
Equine infectious anemia virus enhancer	5' CAGGAA 3'
Lymphotrophic papovavirus	5' GAGGAA 3'

High affinity Fcγreceptor .

This interferon-inducible gene is restricted in expression to the cells of the myeloid lineage (48). Studies on the promoter of this gene lead to the identification of two cis-DNA elements, which confer interferon responsiveness and myeloid cell

activation abilities to the promoter (49). Recent studies have identified that the factor that confers the myeloid cell activating ability is PU.1, which binds to the cis DNA element (49). The binding site for PU.1 in the Fcγ promoter is 5' AGAAAAG 3' and varies considerably from the consensus binding site. This adds Fcγ receptor to the list of myeloid specific genes that are regulated by PU.1.

The promoters described above have all been shown to be regulated by PU.1 binding to the consensus binding site. As can be seen, the binding sites for PU.1 vary considerably from the consensus binding site, and it is impossible to predict the binding of PU.1 to any of its binding sites. For example, the consensus binding site 5' GAGGAA 3' in the CD11b promoter does not bind PU.1 but the sequence 5' GGAGAA 3' binds the protein. Table 1 shows the summary of all the known binding sites for PU.1 from myeloid, B cells, and mast cells. The binding sequence need not necessarily contain any of the consensus nucleotides for the binding site. Many of the uncharacterized myeloid promoters contain consensus binding sites for PU.1, but the direct involvement of PU.1 in the regulation of these genes needs to be demonstrated.

Myeloperoxidase promoter .

The human myeloperoxidase gene was one of the early myeloid specific genes studied. Although the promoter sequence has been well characterized, the functional elements responsible for tissue specific expression of this promoter has not been well characterized (50-53). The promoter contains several PU.1 binding sites, which could serve to regulate myeloid specific expression of MPO, although the direct involvement of PU.1 in the expression of MPO has not yet been shown. The murine myeloperoxidase promoter has been well characterized and consists of multiple functional elements (54). The main regulatory elements responsible for tissue specific expression of murine MPO consists of an E box motif 5' CAACTG 3' at -297 and other multiple enhancer-like elements located between -315 and -241 (54). This region bears considerable homology to the human myeloperoxidase promoter and could be responsible for tissue specific

expression from the human promoter. Recently, the PEPB2/CBF family of oncoproteins has been shown to bind to the enhancer like element 5' AACCACA 3' within the promoter for MPO and regulate expression of the murine MPO promoter (47). Additional studies have focused on the presence of stage restricted DNAse I hypersensitivity sites located upstream of the human MPO promoter (14-16). In addition, differences in methylation pattern around the MPO promoter have been observed suggesting that these two mechanisms could play a crucial role in the overall regulation of the MPO gene (15).

The promoters described above are well characterized in that the *cis* acting DNA elements as well as the *trans* acting binding factors, responsible for myeloid specific activity of the promoter, have been identified. In addition, several myeloid specific promoters have been dissected for functional activity but further characterization of these promoters needs to be done. These include the promoters for Cathepsin G, gp91-phox, CD11c, CD11a, CD13, Granulocyte Colony-Stimulating Factor gene, and the genes for myeloid specific calcium binding proteins, MRP8 and MRP14 (55-61). A brief account of the findings on the functional activity of these promoters follows.

The promoter for Cathepsin G was recently functionally analyzed for their ability to direct tissue and stage restricted expression of the human Cathepsin G in transgenic mice. Cathepsin G, like Elastase gene locus, exists as a cluster of genes on chromosome 14q11.2 (55). When 2.5 kb of 5' flanking region and 0.8 kb of 3' flanking region of the cathepsin G locus were analyzed for activity in transgenic mice, it was found to be sufficient to direct early myeloid specific expression of the transgene in mice (55). The cis acting elements for this promoter have not yet been identified although this promoter contains several Ets binding sites that could serve as targets for PU.1. Similarly the first 1000 bp of the 5' flanking sequences of the genes for CD11c, CD13, MRP8 and MRP14 were found to direct tissue specific expression of the reporter gene in myeloid cells (56-61). In all these promoters, putative binding site for the myeloid specific transcription

factor, PU.1, was present within the active promoter; although, the involvement of PU.1 has not been demonstrated. The promoter for the gp91-phox gene is unusual in that the promoter has been shown to be repressed by the constitutive repressor protein, CCAAT displacement protein (56). The promoter consists of duplicated CCAAT elements that are essential for the regulation of promoter activity. Deletion of the CCAAT elements results in upregulation of promoter activity suggesting that this element directs down regulation of the gp91-phox promoter (56).

The regulation of several avian and murine myeloid specific genes has been studied in greater detail. These include the genes for the murine M-lysozyme, murine mim-1 gene and chicken Myelomonocytic Growth Factor (cMGF) (30, 31, 62-66). The murine M-lysozyme acquires a myeloid-specific and differentiation dependent chromatin configuration, and several DNAse I hypersensitivity sites were detected in the gene domain surrounding the M-lysozyme gene (30, 31). A downstream enhancer directs the myeloid specific demethylation of this gene resulting in myeloid specific expression of this gene (31). The murine mim-1 gene is acted upon by combinatorial activators, c-myb and NF-M, which bind sites in the promoter of this gene (64). C-myb interacts with tissue specific factor NF-M, a c/EBP related myeloid specific factor, to direct expression of this gene only in myeloid cells (62-65). The same tissue specific factor NF-M interacts with AP-1 combinatorially to regulate the expression of avian cMGF by binding to a 47 bp cis regulatory sequence in the cMGF promoter (66). This leads to activation of this gene in myeloid cells.

There appear to be differences in the regulation of myeloid specific genes between different species. For example, c-myb regulates several murine promoters including mim-1, MPO and Elastase, but the involvement of c-myb in the regulation of human myeloid specific genes is yet to be determined. There are several factors, some of which are DNA binding proteins, that have been reported to play a role in the human myeloid differentiation. These include the p53 gene, Bcl-2 gene, c-myc and its interacting

partners mad and max, Id and E box binding bHLH proteins, MZF-1, the gene for the tyrosine kinase c-fes, C and B-myb, C/EBP related factors and the gene for the retinoic acid receptor (67-80).

Expression of wild type p53 gene in promyelocytic leukemic cells, HL-60, induces granulocytic differentiation of HL-60 cells (67). The homozygous deletion of p53 tumor suppressor gene and hence lack of a functional p53 gene occurs in many of the myeloid cell lines including HL-60, L12, K 562 and KG-1a. This may be the primary cause of malignant transformation of promyelocytic cells although p53 null mice show normal development (67, 68). However, the expression of exogenous p53 in HL-60 cells did not cause growth arrest, and p53 is involved in the differentiation process independent of its activity on the cell-cycle (67). Similar results were observed when exogenous p53 was transfected into K 562 cells; this caused partial differentiation of these cells (69). These results suggest that wild-type p53 protein plays a role in hematopoietic maturation by contributing to inhibition of proliferation and induction of differentiation. The summary of all known functions of p53 during normal hematopoiesis has been recently reviewed (70).

Structural analysis of the murine p53 promoter reveals that an E box consensus site CACGTG binds bHLH factors, and the expression of p53 is dependent on bHLH factors. The bHLH domain containing proteins has been involved in the process of differentiation of many cell types and could control proliferation of cells by regulating p53 expression (71, 72). E box binding bHLH proteins have been implicated in myeloid differentiation by the fact that constitutive expression of Id, an HLH repressor protein, in myeloid cells results in the inhibition of differentiation (73). It was observed that the DNA binding activity of the E box proteins increased upon differentiation of myeloid cells presumably due to decreased production of Id protein. This effect may be mediated by the action of bHLH proteins on p53 although several myeloid promoters contain E box motifs. No direct involvement of the E box binding proteins in the activity of myeloid

specific promoters has been demonstrated, and the E box binding proteins may regulate differentiation indirectly (73). However, differentiation of U 937 cells with TPA results in the change in the heterocomplexes between the members of the c-myc family of E box binding proteins (74). In undifferentiated U 937 cells, only Myc:Max complexes were observed, but differentiation results in the formation of complexes between Mad:Max. This switch in heterocomplexes presumably affects genes that are targets for the Myc:Max heterocomplexes and hence may affect cellular proliferation. This suggests that c-myc may play a role in myeloid differentiation, and the expression of c-myc is downregulated upon myeloid differentiation. In fact, HL-60 has been shown to contain amplified c-myc gene in the extrachromosomal double minute chromosome (75). The reduction in the number of the amplified c-myc gene by the addition of hydroxyurea or Dimethyl sulfoxide (DMSO) results in induction of differentiation of HL-60 cells into the granulocytic lineage (75). This suggests that amplified c-myc maintains the undifferentiated and proliferative state of HL-60 cells and clearly shows the role of c-myc in myeloid differentiation.

Bcl-2, another cell cycle related protein known to inhibit induced apoptosis, was examined for its effect on terminal differentiation of myeloid cells (76). Bcl-2 protein levels have been reported to decrease during differentiation of myeloid cells into granulocytes. The effect of constitutive hyperexpression of Bcl-2 in HL-60 cells induced to differentiate with phorbol esters or retinoic acid was examined and it was found that differentiated cells showed prolonged survival unlike control cells that underwent apoptosis (76).

Tsai et al. investigated the role of retinoic acid receptors in the development of neutrophils by introducing a dominant negative mutation of the retinoic acid receptor into multipotential stem cells (77). Myeloid cells can be induced to differentiate by treatment with retinoic acid although the exact molecular mechanisms that mediate this action are not clear. A dominant negative mutation of the retinoic acid receptor results in the block

in neutrophil differentiation at the stage of promyelocytes (77). In addition, one of the chromosomal translocation 15:17, which results in the fusion of the reticnoic acid receptor to the putative transcription factor, PML, leads to a clonal expansion of hematopoietic precursors that are blocked at the promyelocyte stage of differentaition (78). This leads to acute promyelocytic leukemia, and this differentiation block responds well to treatment with retinoic acid. The target of the PML-RAR fusion protein is not clear but the involvement of RAR in the process of neutrophil differentiation is undoubtedly important (79).

The role of c-myb in the regulation of myeloid specific mim-1 has been well characterized (64). To determine the role of c-myb and B-myb in human myeloid cells, antisense oligonucleotides, which inhibit the expression of c-myb and B-myb, were added to cultures of hematopoietic cells (80). After 5 days of culture, significant reduction in the cell growth of U 937 and HL-60 cells were observed. This correlates with the normal pattern of c-myb expression, which closely follows the proliferative capacity of hematopoietic cells (81). A zinc finger transcription factor, MZF-1, preferentially expressed in myeloid cells was recently cloned, and it has been suggested that this gene may play a role in transcription of myeloid genes (9). However, no cellular targets for this gene have been described so far. Another myeloid specific tyrosine kinase gene, c-fes, was implicated to play a major role in the initiation of myeloid differentiation. K 562 cells, transfected with c-fes, acquired the ability to differentiate into the myeloid lineages although the mechanism by which this action is mediated is unclear (82).

Members of the c/EBP family of transcription factor have been implicated in the differentiation of adipocytes and hepatocytes (83). The expression pattern of three different c/EBP proteins in myelononocytic cells was examined by western blot and it was found that the levels of the different proteins varied considerably during differentiation of myelomonocytic cells. These proteins were not detected in the cells of the erythroid or the lymphoid lineage and showed myeloid specificity in expression (84).

The levels of c/EBPα was maximal in the undifferentiated myelomoncytic cells but declined during differentiation towards the granulocytic lineage. The levels of c/EBPδ exhibited a similar pattern but the levels of c/EBPβ increased as the cells differentiated into granulocytes (84). The ability of these proteins to form homo- and heterodimers may allow this family of transcription factors to interact differentially according to state of differentiation and regulate expression of different genes. However, the direct involvement of these genes in the transcription of myeloid specific genes is yet to be shown.

Thus, the analysis of various regulatory factors governing the expression of myeloid specific genes shows that there are multiple levels of regulation mediated by direct and indirect factors. Some of the transcription factors like PU.1 directly bind the promoter sequence to regulate expression. Other factors like retinoic acid receptor and p53, although they are DNA binding factors themselves, regulate the differentiation state of myeloid cells indirectly. An overall picture on how these factors are interrelated in the process of myeloid differentiation is yet to emerge from the studies so far. Further studies aimed at dissecting molecular events that govern expression of myeloid specific genes, and hence differentiation of myeloid cells, will provide information on the relationship between factors that now appear to independently regulate myeloid differentiation.

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A 30 BP ELEMENT IS RESPONSIBLE FOR THE MYELOID SPECIFIC ACTIVITY OF THE HUMAN NEUTROPHIL ELASTASE PROMOTER

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ABSTRACT

Human Neutrophil Elastase (HNE), a serine protease, is expressed only in the promyelocytic stages of granulocyte maturation. We examined several regions of the promoter for transcriptional activity and report that a 30 bp element located between -76 and -106 in the 5' flanking region of HNE is sufficient for myeloid-specific expression of HNE. Gel shift assays using nuclear extracts from myeloid and non-myeloid cells reveal several myeloid-specific complexes binding to the 30 bp element. Examination of DNA-protein interactions shows that at least two myeloid specific proteins of 38 kDa and 55 kDa bind to this element. DNAse I protection analysis reveals two distinct footprints between -80 to -91 and -94 to -104 within this element. Transient expression studies using deletion constructs of the HNE 5' flanking region show that the 30 bp element is active in myeloid cells K 562 and U 937 but not in HeLa cells. Internal deletion of this element results in a 60-85% loss of promoter activity in myeloid cells. Additional functional studies also show that a 19 bp region between -112 and -131 contributes to transcriptional activity of the elastase promoter as well.

INTRODUCTION

Human Neutrophil Elastase (HNE) mRNA is synthesized in the promyelocytic stages of normal bone marrow myeloblast maturation and the protein is stored in the azurophilic granules of mature neutrophils. HNE is a potent elastinolytic and collagenolytic protease that functions in tissue turnover and host defense mechanisms. The expression of HNE mRNA is restricted to the promyelocyte stage of granulocyte maturation and shows tissue and stage-specificity (1). The HNE gene has been cloned and it has been shown that the regulation of expression is mainly controlled at the transcriptional level (2,3). Transcriptional analyses using nuclear run-on assays showed that HNE mRNA appears to be transcriptionally down-regulated upon differentiation of HL-60 cells (3). In preliminary studies using promoter constructs, we have shown that the 5' flanking region of HNE has transcriptional activity (4). HNE mRNA is expressed

in promyelocytic cell-lines HL-60, PLB-985 as well as in the myelomonocytic U 937 cell-line, and differentiation of these cells with phorbol esters rapidly down-regulates HNE mRNA expression (5,6).

Several other granulocyte-specific proteases are expressed at the promyelocytic or promonocytic stage of myeloid differentiation. So far, five human genes, Azurocidin, Proteinase 3, Neutrophil Elastase, Cathepsin G and Myeloperoxidase, have been cloned (2,7-10). Interestingly, analysis of the promoter structure of these five genes reveals several conserved features including TATA-like elements, CCAAT box, a PU.1 binding site and a C/T rich element within the first 200 bp of the major transcriptional start site. The *cis*-acting elements responsible for myeloid-specific expression of these genes have not yet been described. There are two different groups of serine proteases located as clusters of genes: The Elastase cluster of serine proteases is located on chromosome 19 pter and the cathepsin G family of serine proteases on chromosome 14q11-12 (11,12). Regulatory mechanisms such as locus control regions and DNAse hypersensitivity sites, identified in the β-globin locus, have not yet been characterized so far in the serine proteases gene clusters (13,14). Preliminary evidence suggests that the myeloperoxidase (MPO) gene is regulated by DNA methylation and by DNAse hypersensitivity sites located upstream of the gene (15,16).

Some of the elements responsible for transcriptional regulation of genes that are expressed at more mature stages of granulocyte differentiation have been identified. The promoters for some of these genes including the CD11b, CD18, gp91phox, CD11c and M-CSFR have been characterized by transient transfection studies (17-23). In the CD11b and M-CSFR genes, the binding of PU.1 protein to the core sequence GAGGAA has been shown to be critical for tissue specific expression (17,23). In addition, Sp1 binds to the CD11b promoter specifically *in vivo* to activate transcription (18). In the CD18 promoter, PU.1 and another Ets-related factor GABP have been implicated in the tissue-specific regulation of expression (20). In gp91 phox, a duplicated CCAAT box present in

the promoter binds differentially regulated CCAAT-displacement protein and regulates expression (21). Myeloid-specific expression has also been characterized in the chicken mim-1 gene (24). Here two factors, c-myb and a c-EBP like factor NF-M, act as combinatorial activators of myeloid-specific mim-1 expression (26,27). In murine granulocytopoiesis, at least three different sets of genes, Id and its interacting bHLH proteins, c-myc and its associated factors, and retinoic acid receptor proteins, have been shown to play a role in myeloid differentiation (26-28). These studies suggest that the regulation of gene expression during myeloid maturation requires interaction of numerous *trans* acting factors. The putative binding sites for Sp1, PU.1, c-myb, multiple E-box motifs capable of binding bHLH proteins and a duplicated CCAAT box can also be found within 1000 bp of the HNE transcriptional start site.

Here we characterize the 5 ' flanking region of HNE and report that a 30 bp element present within the first 150 bases upstream from the transcriptional start site is sufficient to direct myeloid-specific expression of the HNE gene. This element has homology to sequences in the promoters of other promyelocyte-specific genes suggesting that it may play a role in the regulation of other myeloid-specific genes.

MATERIALS AND METHODS

Preparation of Nuclear Extracts-- U 937, HL-60, PLB-985 and K 562 cells were grown in suspension in RPMI-1640 media supplemented with 10% fetal calf serum (GibcoBRL) and antibiotics to a density of $5x10^5$ cells/ml. HeLa and Hep G2 cells were grown in DMEM media containing 10% fetal calf serum and antibiotics to near confluence. 10^9 cells were harvested by centrifugation and nuclear extract was obtained as described by Dignam *et al.* with the following modifications: protease inhibitors aprotonin, PMSF, leupeptin and pepstatin were added at appropriate concentrations (29). Aliquots of nuclear extracts were stored frozen at -70 ^{0}C and the protein concentration in these preparations were in the range of 6-8 mg/ml.

Electrophoretic Mobility Shift Assays (EMSA)- The probes used for the EMSA were synthesized on an oligonucleotide synthesizer. The sequence of the 30 bp oligonucleotide duplex is as follows: 5' GCC CTG TGC CAG GGG AGA GGA AGT GGA GGG 3'. In order to create oligonucleotide duplexes, complementary oligonucleotides were annealed, gel purified and end labeled with ^{32}P γATP and T4 Polynucleotide Kinase. Twenty micrograms of nuclear extract was added to binding buffer containing 10mM Tris HCl (pH 7.5), 50mM KCl, 5mM $MgCl_2$, 1mM DTT, 1mM EDTA, 12.5% glycerol and 0.1% Triton X-100 with 3 μg of poly(dI-dC). The reaction was preincubated for 20 min. at 4 ^{0}C. Then, 1 ng of labeled probe was added and incubation continued for another 20 minutes. Limited proteinase K digestions were carried out for 5 min. at 37 ^{0}C using enzyme concentrations from 100 to 1000 ng (30,31). Cold competition experiments were performed by adding 100-fold molar excess of unlabeled duplex oligonucleotides after the addition of labeled probe. Antibody supershifts were carried out by adding 5μl of anti-amino terminus peptide PU.1 antiserum (a kind gift of Dr. R. Maki) to the binding reactions. The reaction was then loaded onto a 6% or a 10% high ionic strength non-denaturing polyacrylamide gel containing 0.5 X TBE with 2% glycerol. The products were resolved and visualized by autoradiography.

DNAse I Footprinting Assay- A 201 bp fragment of the HNE promoter between +7 and -196 was subcloned after PCR into the PCRII vector (Invitrogen). Multimers of the 30 bp element were made by annealing and ligating 30 bp duplexes with 5' overhangs and the products were subcloned into pT_7T_3 (Pharmacia) at the Sma I site. The 201 bp fragment and the pentameric clone of the 30 bp element were used as substrates for the DNAse I protection assays. Singly end labeled probes were prepared by restriction digestion with appropriate enzymes, end-labeled with T4 kinase and ^{32}P γATP, and digested again with a third enzyme to release the probe labeled at only one 5' end. One nanogram of the purified probe, labeled at only one 5' end, was used in the binding reactions containing 25mM Tris HCl pH 8.0, 50mM KCl, 6.5mM $MgCl_2$, 0.5mM EDTA,

10% glycerol and 0.5mM DTT and 80-100 µg of nuclear extracts. After binding for 20 min. on ice, 5mM $CaCl_2$ and 10mM $MgCl_2$ were added and 0.05 units of RQ1 RNase-free DNAse 1 (Promega) were added at room temperature for exactly 1 min. The reaction was stopped by addition of 0.1M NaCl, 15mM EDTA and 1% SDS. The digested probe was purified by phenol extraction and ethanol precipitation. The products were resolved on a 6% acrylamide sequencing gel and autoradiographed.

South-Western Blot- Fifty micrograms of crude nuclear extracts from myeloid and non-myeloid cells were resolved on a 10% SDS-PAGE denaturing gel. The gel was run at 4 ^{0}C overnight at 10mA and then equilibrated in transfer buffer containing 25mM Tris, 192mM glycine and 15% methanol. The proteins were electroblotted onto Immobilon-P filters (Millipore) at 10 V/cm for 3 h. The proteins were denatured in 6M guanidine hydrochloride solution in TNE-50 (10mM Tris HCl, 50mM NaCl, 1mM EDTA and 1mM DTT) for 5 min. at room temperature. The filters were renatured by 2-fold dilution of the guanidine hydrochloride with TNE-50 over 30 min and blocked with 5% non-fat dry milk in TNE-50 for 1 h at room temperature. The filters were washed in TNE-50 buffer and probed in TNE-50 containing 10 ng/µl (low) or 100 ng/µl (high concentration) of poly (dI-dC) DNA and 200 ng of purified end labeled probe in 3 ml of binding solution for one h at room temp. The filters were washed in three changes of TNE-50, dried and autoradiographed.

Transfections- The reporter vectors for assay of promoter activity were constructed by subcloning PCR fragments of appropriate promoter deletions into the Sma I site in the pUMSCAT vector (a kind gift of K.Kurachi). The internal deletion of the 30 bp element from the -153 CAT vector was created by site-directed mutagenesis using the Sculptor *in vitro* mutagenesis kit (Amersham). Briefly, mutant oligonucleotides were annealed to single-stranded template and the mutated strand synthesized under positive selection. The mutations were then verified by sequencing. Twenty micrograms of reporter test plasmid were precipitated in ethanol along with 0.5 µg of pCMV-Luciferase construct

used as an internal control for transfection efficiency. 2 X 10^7 U 937, K 562 or HeLa cells in logarithmic growth phase were harvested and resuspended in 700 µl of serum-free RPMI-1640 media. Plasmids were resuspended in 100 µl of serum-free RPMI-1640 media and added to electroporation cuvettes (BioRad) containing the cells. The cuvettes were placed on ice for 5 min. and then electroporated at 300 V and 960 µF (Gene pulser, BioRad). Following electroporation, the cells were placed on ice for 15 min. and transferred to flasks with RPMI-1640 media containing 20% fetal calf serum. After 48 h the cells were harvested by centrifugation and resuspended in 400 µl of 1X reporter lysis buffer (Promega). The cells were lysed and the protein content in the cell-lysate was determined by BCA protein assays (Pierce). Equal amounts of protein (usually 200 µg) were used to perform Chloramphenical Acetyl Transferase (CAT) assays as per manufacturer's instruction (Promega). The amounts of CAT in the lysates were estimated by liquid scintillation and corrected for transfection efficiency by standardization to luciferase levels determined on the same lysates (Promega). At least three independent transfections in duplicate were used to determine the reporter activity of different constructs.

RESULTS

EMSA Shows That Hematopoietic Cell-lines Contain Factors That Bind to the 30 bp Eement- The sequences within the 30 bp element was found to be conserved among different promyelocyte-specific promoters and this sequence conservation led us to test the hypothesis that this sequence is involved in the myeloid-specific regulation of the HNE promoter. We performed EMSA with nuclear extracts from myeloid and non-myeloid cell lines to determine if myeloid-specific factors bind specifically to the 30 bp region between -76 and -106. We found that nuclear extracts from promyelocytic cell lines PLB-985 and HL-60 consistently produced multiple DNA-protein complexes labeled as complexes A to F (Fig. 1, lanes 4, 5). In contrast, U 937 and K 562 extracts only produced complexes corresponding to shifts A, B, C and D observed in PLB-985

and HL-60 extracts (Fig. 1, lanes 3, 6). Besides the differences in the number of complexes formed, there were also significant differences in the intensity of the bands observed even though the nuclear extracts contained similar amounts of proteins as assayed by coomassie staining (data not shown). The intensity of complexes B, C and D observed in U 937 and K 562 extracts were much lower than those observed in PLB-985 or HL-60 extracts. These observations could reflect differences in the concentration of DNA binding proteins present in these cell lines. No shifts were observed with HeLa (Fig. 1, lane 7) or Hep G2 (data not shown) nuclear extracts.

Interestingly, complexes E and F were observed in promyelocytic cell-lines PLB-985 and HL-60 but not in other hematopoietic cells like U 937 and K 562. Several different mechanisms, including post-translational modifications, proteolytic cleavage and the existence of novel promyelocyte-specific cofactors, could give rise to the additional complexes observed only in extracts of promyelocytic cells. Since myeloid nuclear extracts are rich in proteases, we investigated the possibility that the different complexes observed in the nuclear extracts of promyelocytic cells were protein degradation products by performing proteolytic clipping band-shift assays (PCBA) on these extracts (30,31). This assay is based on the premise that if lower molecular weight complexes are degradation products, then limited proteolysis performed on the gel-shifted bands will result in the accumulation of lower molecular weight complexes as more of the precursor complexes are proteolytically clipped. Limited proteinase K digestions on the shifted complexes A to F failed to increase the signal of the lower molecular weight complexes E and F as the higher complexes were degraded and no additional bands appeared upon digestion (Fig. 2). Proteinase K digests proteins non-specifically and possibly digests all complexes equally so there may be no preferential accumulation of digested products. To rule out artifacts based on this possibility, we substituted Trypsin, which specifically cleaves after lysine or arginine residues, for proteinase K and similar

results were obtained (data not shown). These experiments suggest that the low molecular weight complexes observed in the band-shift assays are not proteolytic cleaved products.

We used the polyanionic detergent, Sarkosyl, at varying concentrations to determine if the proteins responsible for complexes A to F observed in promyelocytic extracts had different binding affinities. Sarkosyl interferes with hydrophilic interactions and acts as a non-specific competitor in these reactions. We observed that complexes B and D appeared as weak bands and hence had weak DNA-binding properties when compared to the other complexes. Complexes A, C, E and F bound to the probe in the presence of 0.06% Sarkosyl indicating that these complexes have similar high binding affinities (Fig. 3). As shown in Fig. 3, lanes 8 and 9, concentrations of Sarkosyl greater than 0.06% resulted in the marked decrease in the formation of complexes B to F. Complex A was affected by Sarkosyl concentrations to a lesser degree indicating that this complex has higher DNA-binding affinity than the other complexes. These observations suggest that different complexes display different DNA-binding affinities. This finding is consistent with the results of PCBA and indicates that different proteins are responsible for complexes A to F observed in the EMSA.

As shown in Fig. 4, we have examined the sequence specificity of the observed complexes and determined the extent of the binding sites within the 30 bp element by performing cold competition EMSA using a variety of mutated and partially deleted duplex oligonucleotides. These studies demonstrate that the 30 bp duplex oligonucleotide (-106 to -76), as well as the deleted oligonucleotides 30C1 (-102 to -76) and 30C2 (-106 to -80), compete effectively with the wild-type probe for the formation of all of the complexes observed in the presence of PLB-985 extracts (Fig. 4). This indicates that the sequence between -102 and -80 is the minimal essential region sufficient for complete competition. Shorter oligonucleotide duplexes AB (-92 to -82) or 10 (-102 to -82) did not compete for binding (Fig. 5). Interestingly, oligonucleotide AB

contains the PU.1/Ets binding motif but not adjoining sequences, while oligonucleotide 10 contains all the essential sequences defined above except two G residues at positions 80 and 81. This suggests that the two G residues at positions 80 and 81 are crucial for binding and competition. Duplex oligonucleotides representing the sequence between -106 and -96 did not affect binding or competition (Fig. 5). Duplex oligonucleotides containing irrelevant sequences also failed to compete for any of the shifted bands (data not shown). The sequences of the oligonucleotides used in competition experiments as well as their binding activities and ability to compete away the EMSA complexes are described in Fig. 5.

DNAse I Protection Analysis of the 30 bp Element Reveal Myeloid-Specific Footprints- DNAse I protection analysis was performed on the first 200 bp of the HNE 5'-flanking region in the presence of nuclear extract from the Hela and various hematopoietic cell lines. Identical amounts of nuclear extracts from PLB-985, U 937, HL-60 and HeLa cells were used in these studies. We observed that the "TATAAG" element between -31 and -24 was protected from digestion equally by all of the nuclear extracts (Fig. 6a). No footprints were observed around the putative binding sites for Sp1 (-12 to -18), c-myb (-47 to -53) or the putative "CCAAT" element (-55 to -63). In contrast, the entire 30 bp region between -76 and -106 was protected from digestion with PLB-985 and HL-60 nuclear extracts but not with HeLa extract (Fig. 6a). Similar protections were also observed with U 937 extracts (data not shown). The position of the 30 bp element within the 200 bp promoter fragment prohibited direct determination of the exact sequence protected from DNAse I digestion. In order to examine this region more effectively, a concatemerized probe consisting of five repeats of the 30 bp element was subcloned and used for protection analysis. It has been shown that multimerized probes have increased affinity for DNA-binding proteins and are, therefore, better substrates for analyzing DNAse I protection patterns (32). As shown in Fig. 6b, two sequences on the anti-sense

strand corresponding to (-104) 5' CCTGTGCCA 3' (-96) and (-91) 5' GAGAGGAAGTGG 3' (-80) were protected from digestion in the presence of PLB-985 myeloid nuclear extracts but not in the presence of HeLa nuclear extracts. In addition, a DNAse hypersensitivity site was observed at the G residue in position -93 that may reflect protein-induced bending of DNA. Additional DNAse I protection analysis was also performed on the adjoining sequences of the HNE 5' 200 bp, and Fig. 7 shows the overall summary of the footprints observed within the first 200 bp of the HNE promoter.

At Least Two Different Myeloid-Specific Proteins Bind to the 30 bp Element- Nuclear extracts from promyelocytic cell lines, HL-60 and PLB-985, and the hepatoma cell line Hep G2 were separated on an SDS-PAGE, transferred onto Immobilon-PVDF membranes and bound with the radiolabeled duplex 30 bp element. Three major bands representing proteins of 38, 42 and 55-60 kDa appear to bind to the 30 bp element with high specificity in the presence of 100 ng/µl poly(dI-dC) in myeloid cells (Fig. 8). In addition, proteins of 30 kDa and 80-90 kDa also bound to the 30 bp element with lesser affinity. The signal from these proteins was of lower intensity and difficult to identify at higher concentrations of poly(dI-dC) (Fig. 8). However, these bands were observed to increase in intensity when lower concentrations of poly(dI-dC) were used (data not shown). Of these, the 42 kDa protein was also observed in nuclear extracts from Hep G2 (Fig. 8).

We probed the nuclear extracts from these cells with a GA rich probe, similar in GA content to the 30 bp probe but without the consensus Ets binding site in the presence of low concentrations of poly (dI-dC). This probe does not bind any myeloid specific factors when used in EMSA studies. Fig. 9 shows that there are two proteins of 42 and 80-90 kDa that bind to GA rich probe suggesting that these proteins recognize GA rich sequences. The other myeloid-specific proteins of 30, 38 and 55-60 kD did not bind to

this probe suggesting that these proteins specifically recognize the 30 bp probe. These experiments demonstrate that the 30 bp element binds at least two different myeloid-specific proteins of 38 and 55-60 kDa.

The 30 bp Element is Sufficient to Confer Myeloid-Specificity to the HNE Promoter- To establish the functional significance of the myeloid-specific factors binding to the 30 bp element, promoter deletion constructs in CAT reporter plasmids were transfected into both myeloid and non-myeloid cells. U 937 and K 562 cells were chosen as myeloid cell lines because these cells are readily transfectable than HL-60 and PLB-985 promyelocytic cells (33). In our experience, the transfection efficiency in PLB-985 and HL-60 cells was two orders of magnitude less than U 937 cells. K 562 cells, although considered to be a model for erythroid differentiation, are also capable of differentiation into granulocytes and megakaryocytes upon exposure to differentiating agents like phorbol esters and retinoic acid (34,35). This suggests that K 562 cells may serve as a useful early hematopoietic cell model in the study of myeloid specific gene expression (34). As non-myeloid controls, HeLa cervical carcinoma cells were used, as these cells express neither HNE nor PU.1. All experiments were repeated at least three times independently and the results were normalized for equal transfection efficiencies by using luciferase as an internal control.

These studies showed that the first 106 bases of the 5' flanking region, containing only the TATA box, CCAAT box and the 30 bp element, was sufficient to direct myeloid-specific expression of the CAT reporter gene in U 937 (Fig. 10a) and K 562 cells (Fig. 10b). In contrast, there was no expression of reporter gene in HeLa cells (Fig. 10c). The CAT activity of promoterless vector in the myeloid cells was equal to about 1% CAT acetylation and the CAT activity of the -106 CAT construct represented about 2% CAT conversion as determined by the liquid scintillation counting assay. The highest activity in myeloid cells was observed with the -153 CAT construct equivalent to 2.25-fold increase over promoter-less construct. The CAT activities for the various promoter

constructs are reported as percentage increase over promoterless plasmid with maximal activity of 100% assigned to the -153 CAT construct (Fig. 10 a,b,c). The results were similar for U 937 and K 562 transfections. The internal deletion of the 30 bp element from the -153 CAT construct results in a 60-90% decrease in myeloid-specific reporter gene expression in both K 562 and U 937 cells. These results suggest that the 30 bp element is critical in directing myeloid-specific expression of HNE. It was also observed from these transfection studies that the -131 CAT construct has increased activity over the -106 CAT construct indicating that the 19 bp element between -112 and -131 may contribute to transcriptional activity. However, the 19 bp element does not contribute to transcriptional activity in HeLa cells suggesting functional cooperativity between the 30 bp element and the 19 bp element only in myeloid cells. These studies also show that there are no other significant *cis*-acting elements that function as activators of transcription within the first 1000bp that we have examined so far (Fig. 10). The -1024 construct does not show any significant increase in CAT activity over the -106 CAT construct. The low level of promoter activity, consistently seen (n=9) in this promoter, may be attributed to the weak activation abilities observed with PU.1 binding sites. In cotransfection studies with multiple PU.1 binding sites, Klemsz *et al.* observed only a 3-4-fold increase in activity over basal promoter activity (36).

One of the Complexes Interacts With the Antibodies Against PU.1- Since the 30 bp element contains the core PU.1 recognition sequence, we have examined the interaction between antibody against an amino terminus peptide of PU.1 and the complexes observed in EMSA using myeloid extracts. This antibody abolishes the binding of complex C and supershifts it to higher molecular weight complex (Fig. 11). Normal serum does not interact with any of the complexes found in the nuclear extracts. This suggests that EMSA complex C is due to the interaction between PU.1 and the 30 bp element. The protein(s) responsible for the other complexes observed in these experiments remain to be identified.

DISCUSSION

Sequence similarity exists between the elements in the HNE promoter characterized in the present study and elements found in the promoter of other promyelocyte-specific genes. All of the promyelocyte specific promoters examined so far contain a "TATA" element. In contrast, the promoters of myeloid genes expressed at later stages of granulocyte /macrophage maturation, such as CD11b, CD18 and CD11c, lack the "TATA" box. The Ets/PU.1 binding site found in the 30 bp element is also present in the promoters of proteinase 3 (-80), azurocidin (-184), cathepsin G (-15, -39, -90), myeloperoxidase (MPO) (-94, -56), mouse neutrophil elastase (-93) and in the 3' flanking region of human MPO (13896) (37). Another protein binding segment of the 30 bp element identified by DNAse I protection studies is the sequence -104 CCTGTGCCA -96. An inverted repeat of this element is found in the HNE promoter (-34) and elements with similarity to this sequence can also be found in the promoters of proteinase 3 (-222), azurocidin (-199), myeloperoxidase (13880) and mouse neutrophil elastase (-104 and -83). Conservation of these sequences among several members of the promyelocyte-specific protease family suggests a functional role. Preliminary studies from another laboratory suggest that the 30 bp element in the 3' flanking region of MPO and the 19 bp element in the 5' flanking region play a role in achieving maximal expression of the myeloperoxidase gene (38).

EMSA competition studies show that the sequence -102 TGTGCCAGGGGAGAGGAAGTGG -80 is essential for factor binding. The removal of two G residues at positions 80 and 81 from the 3' end in the oligo-duplexes AB and 10 remarkably affected factor binding. Interestingly, the sequence AB containing the core PU.1 binding site 5' GAGAGGAAGT 3' was not sufficient for binding or competing any of the mobility shifts observed with myeloid extracts. The bases surrounding the PU.1 recognition site have been shown to be critical for PU.1 binding. The normal consensus binding site for PU.1 has been established as 5' AAAGGGGAAGTG 3' and it has been

reported that mutation of the non-core A residues to C residues abolishes PU.1 binding (17,39,40). The PU.1 binding sequence in the HNE promoter, 5' GGGGAGAGGAAGTGG 3', varies considerably from the consensus in its surrounding sequences and is similar to the PU.1 sequence present at -134 in the CD11b promoter, which does not bind PU.1 (17). This sequence may be recognized by other Ets-related proteins that bind to the GGA core sequence. We have shown that PU.1, a B cell and macrophage-specific transcription factor, which migrates as different species of 37-44 kDa, represent one of the myeloid-specific gel-shifts (Complex C) and could correspond to the 38 kDa band seen in southwestern blots probed with the 30 bp element. Recently, members of the GABP family of Ets-related factors, which recognize the sequence CGGAR, have been implicated in the regulation of the CD18 gene (20). GABPα and GABP migrate as 55 kDa and 80 kDa protein, respectively, and may recognize this sequence within the 30 bp element (41). Other members of the Ets super family of proteins such as Spi-B also recognize the core PU.1 sequence but the complete consensus binding site for this transcription factor has not been identified (42). A G+A rich probe is also recognized by multiple proteins in the nuclear extracts of myeloid and non-myeloid cells by south-western blot analysis. Since several Ets-related proteins recognize and bind PU.1 boxes, identification of the PU-box binding proteins present in nuclear extract of promyelocytic cells is the subject of further investigation.

The first 106 bases of the HNE promoter direct expression of a reporter gene in U 937 and K 562 cells but not in HeLa cells. An important common feature of U 937 myelomonocytic cells and K 562 chronic myelogenous leukemic cells is that both express PU.1 (43). U 937 cells appear to aberrantly express HNE mRNA although not at the same levels as HL-60 and PLB-985 cells. K 562 cells may represent early myeloid/erythroid blasts capable of differentiation into myeloid and erythroid lineages (34). Since HNE mRNA is expressed specifically during the promyelocytic stage of granulocyte maturation, K 562 would not be expected to express HNE mRNA. The

promoter is active in both U 937 and K 562 cells suggesting that the 30 bp element is responsible for myeloid specific expression but not sufficient to direct stage-restricted expression of HNE. If PU.1 is involved in the regulation of genes like CD11b, CD18 and HNE that are expressed at different stages of granulocyte maturation, the mechanisms by which this regulation occurs are undoubtedly complex. Recently, PU.1 has been shown to activate transcription in the immunoglobulin κ light chain 3' enhancer by interaction with a novel B cell-specific coactivator, NF-EM5 (40,44). It is possible that different tissue-specific "coactivators" similar to NF-EM5 are present in the nuclear extracts of myeloid cells and these interact with PU.1 to determine overall regulation of different genes. These observations acquire significance due to the multiple complexes observed in EMSA using the 30 bp element. It is possible that complexes A and B arise from heteromeric interactions between proteins that bind to the 30 bp element.

U 937 and K 562 nuclear extracts differ significantly from promyelocytic extracts in the number as well as in the intensity of the complexes observed by EMSA. Our preliminary studies indicate that the complexes are independent moieties and not proteolytically related to one another. We propose that there may be differential processing of factors or the presence of still uncharacterized promyelocyte specific factors (similar to Spi-B) which are responsible for the differences observed. PU.1 is a likely candidate for differential processing as it can undergo multiple phosphorylation events (40,44). The differences in the intensity of the bands may also reflect on the concentration of PU.1 and related factors in these cells used in these studies. The promyelocytic cell lines HL-60 and PLB-985 have higher concentrations of PU.1 and related factors than U 937 and, therefore, produce more intense EMSA bands and larger amounts of HNE mRNA. The mechanisms that mediate the stage-restricted promyelocytic/promonocytic specific expression of HNE mRNA remain unclear, and it is apparent that additional regulatory sequences and nuclear factors will have to be identified before for the overall developmental regulation of HNE is fully understood.

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Fig. 1. EMSA analysis of various myeloid and non-myeloid nuclear extracts binding to the 30 bp element. Twenty micrograms of various extracts were used in the binding reactions and loaded on the gel in the following order: no extract, PLB-985 extract with no poly(dIdC) competitor, U 937, PLB-985, HL-60, K 562 and HeLa. The various shifts observed in different cells are indicated as complexes A to F. Complexes A, B, C and D were observed in U 937, PLB-985, HL-60, and K 562 nuclear extracts and complexes E and F were observed in HL-60 and PLB-985 extracts only. The complexes B and D observed in U 937 and K 562 extracts were of lighter intensity. The position of the free probe is also indicated. After the binding reactions are complete, the products were resolved on a 10% non-denaturing gel and autoradiographed.

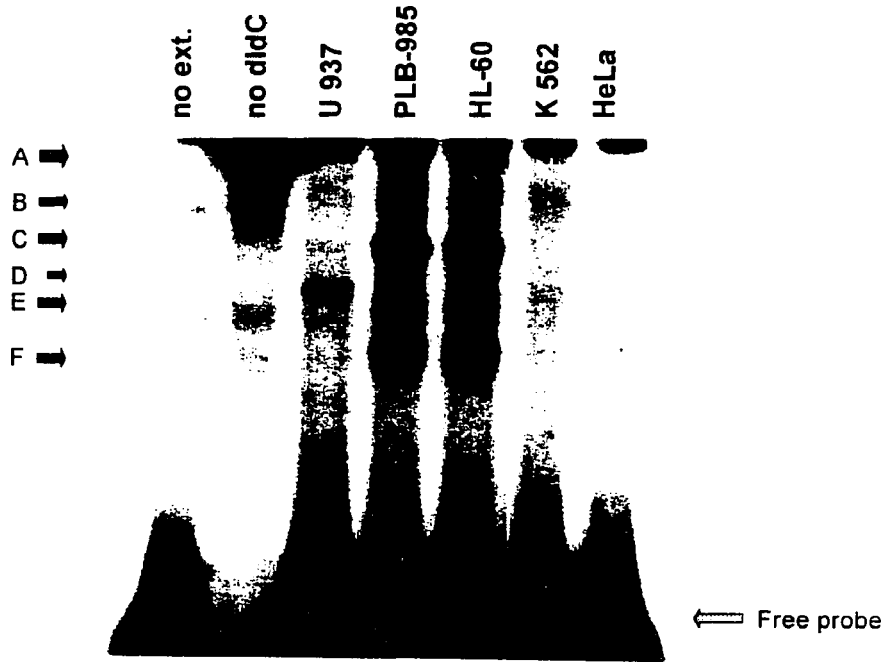

no ext.
no dIdC
U 937
PLB-985
HL-60
K 562
HeLa
A
B
C
D
E
F
Free probe

Fig. 2. Limited proteinase K digestions of the EMSA binding reactions. Twenty micrograms of PLB-985 nuclear extract in binding buffer was digested with varying amounts of proteinase K for 5 min. at 37°C. The amounts correspond to 0 ng (lane 3), 50 ng (lane 4), 100 ng (lane 5), 250 ng (lane 6), 500 ng (lane 7), 750 ng (lane 8) and 1000 ng (lane 9) of enzyme. The digestions were resolved on a 10% (0.5X TBE) non-denaturing gel, dried and autoradiographed. The arrows indicate the various complexes A to F observed with the 30 bp probe. Free probe and the top of the gel are indicated in the fig. No extract control (lane 1) and PLB-985 (lane 2) are also indicated in the fig. Complexes E and F do not accumulate and increase in intensity as all the other complexes are degraded. Note that the autoradiograph was exposed for half the time of exposure as Fig. 1a and hence complexes B and D were not clearly identifiable.

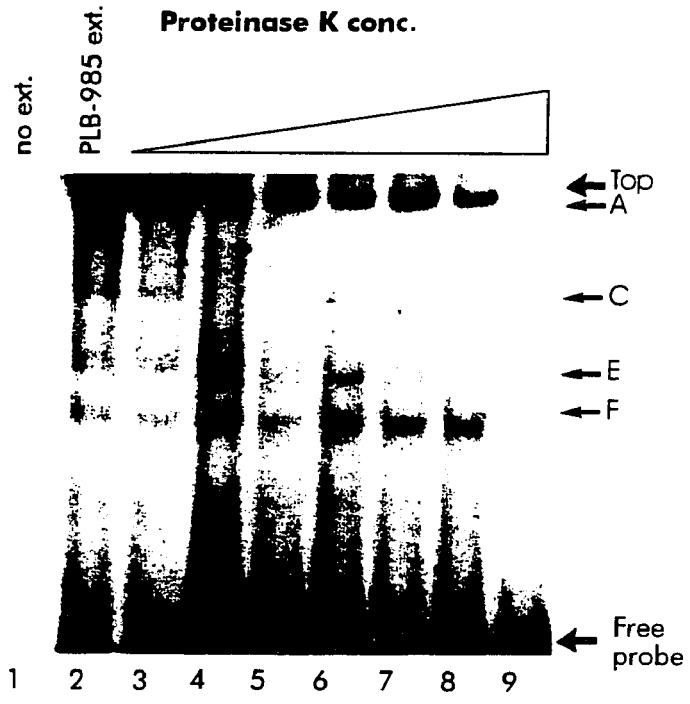

Proteinase K conc.
no ext.
PLB-985 ext.
Top
A
C
E
F
Free
probe
1
2
3
4
5
6
7
8
9

Fig. 3. Sarkosyl competitions of the EMSA binding reactions. Sarkosyl, at final concentrations of 0%, 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06% and 0.1% corresponding to lanes 2 to 9, was added to the binding reactions containing 20 μg PLB-985 nuclear extracts. After the completion of incubation at 4 °C, the products were resolved on a 10% non-denaturing gel and autoradiographed. Gradual loss of binding activity was observed as the Sarkosyl concentrations were increased. Abolition of binding of complexes C, D, E and F occurred at about 0.1% Sarkosyl concentration indicating that these complexes displayed identical binding affinities. Complex A was observed to bind to the probe although with less affinity at 0.1% Sarkosyl. The top of the gel and the free probe are indicated.

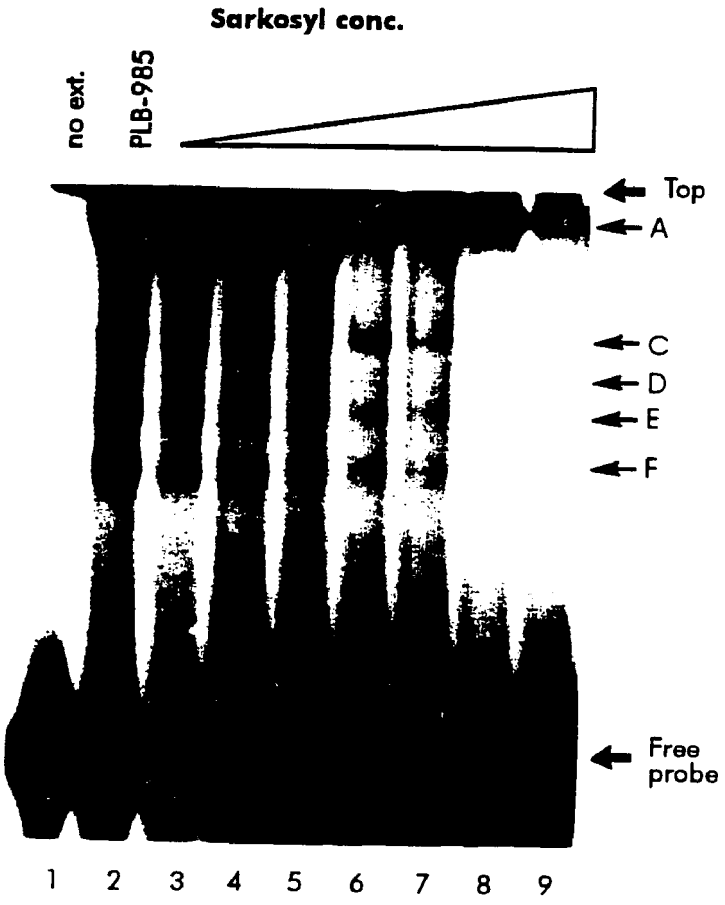

Sarkosyl conc.
no ext.
PLB-985
Top
A
C
D
E
F
Free probe
1 2 3 4 5 6 7 8 9

Fig. 4. Cold competition EMSA using the 30 bp element. One hundred-fold molar excess of various unlabeled probes, corresponding to sequences shown in Fig. 2b, were added to the binding reactions containing PLB-985 extracts after the addition of labeled probe. The reactions were then loaded on a 6% non-denaturing gel, electrophoresed and autoradiographed. The oligonucleotide duplexes 30 bp (lane 3), 30C1 (lane 4) and 30C2 (lane 5) added in 100-fold excess competed the complexes B to F. The complex A, shown in Fig. 1c to have increased DNA affinity, needed 200-fold excess cold duplexes to completely compete away the binding. Note that the percentage of the gel is 6% acrylamide in order to resolve complexes A to F better. The common sequence between these three oligonucleotides is the sequence 5' TGT GCC AGG GGA GAG GAA GTG G 3', which is sufficient for complete competition. No extract (lane 1), Control PLB-985 extract (lane 2), top of the gel and free probe are indicated.

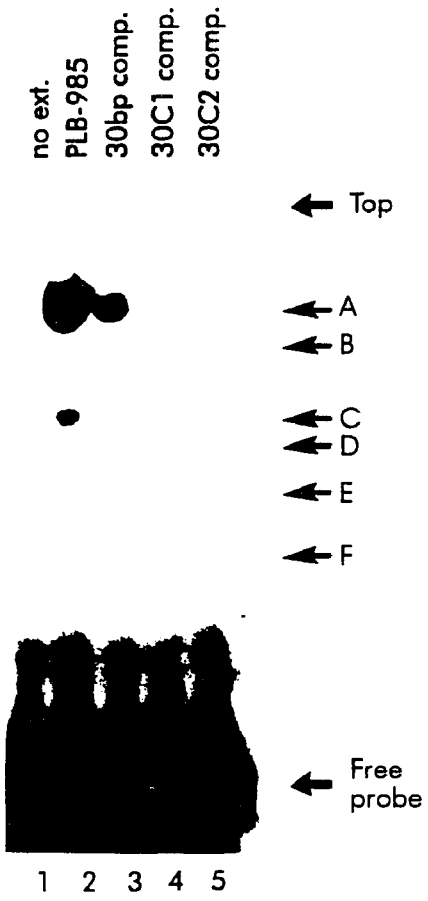

no ext.
PLB-985
30bp comp.
30C1 comp.
30C2 comp.
Top
A
B
C
D
E
F
Free probe
1 2 3 4 5

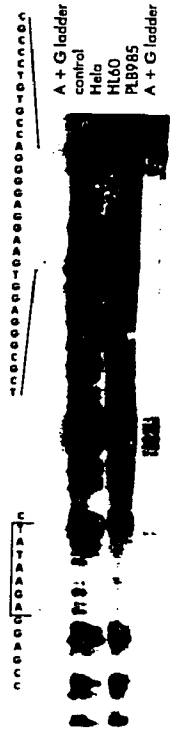
Fig. 5. Sequences of competitors used in EMSA analyses. Various fragments of the 30 bp element designated as AB, CD, 10, 30C1, and 30C2 were used in EMSA binding and cold competition experiments. The results were tabulated and the relative ability of the oligo-duplexes to bind or compete indicated on a scale from ± to +++. Probes 30 bp, 30C1 and 30C2 were effective in binding and competition whereas probes 10, AB and CD were not effective. The probe designated 10 contained the sequence 5' TGT GCC AGG GGA GAG GAA GT 3' but failed to compete in the EMSA experiments suggesting that the two residues at -80 and -81 may be critical for binding of the different complexes.

Probe	Sequence	EMSA	Competition
	-106 -76		
30 bp	GCCCTGTGCCAGGGGA**GAGGAA**GTGGAGGG	+++	+++
AB	GA **GAGGAA**GT	+/-	+/-
CD	GCCCTGTGCC	+/-	+/-
10	TGTGCCAGGGGA**GAGGAA**GT	+/-	+/-
30C1	TGTGCCAGGGGA**GAGGAA**GTGGAGGG	+++	+++
30C2	GCCCTGTGCCAGGGGA**GAGGAA**GTGG	+++	+++

Fig. 6 The DNAse I protection assays of a) The region between +7 and -106. Eighty micrograms of HeLa, HL-60 and PLB-985 nuclear extracts were added in binding reactions to a anti-sense strand end-labeled probe. DNAse I was added to the reactions for 1 min. and the digestions stopped by organic extractions. The products were loaded onto denaturing sequencing gel, resolved and autoradiographed. The +7 end is at the bottom of the gel and the -196 end at the top of the gel. The position of the TATA box, the G+A ladder and the 30 bp element is also indicated. The control and HeLa lanes are identical and run together except for the protection seen at the TATA box in the HeLa lane only. The TATA box and the 30 bp element were protected in PLB-985 and HL-60 extracts. Control lanes indicate reactions in which no extracts were added to protect the probe. b). The 30 bp concatemerized probe in the anti-sense strand. The probe is labeled in the anti-sense strand and is in the same orientation as in Fig. 3a. The -76 end of the 30 bp element is at the bottom of the gel and the -106 end at the top of the gel. Two regions of protection indicated by bars and the hypersensitive site (arrow) observed only in PLB-985 extracts are indicated in the figure. HeLa extracts do not afford any protection from digestion.

A

DNase I Footprinting of the region between -1 and -200 anti-sense strand



B

DNase I Footprinting on the 30bp Element anti-sense strand

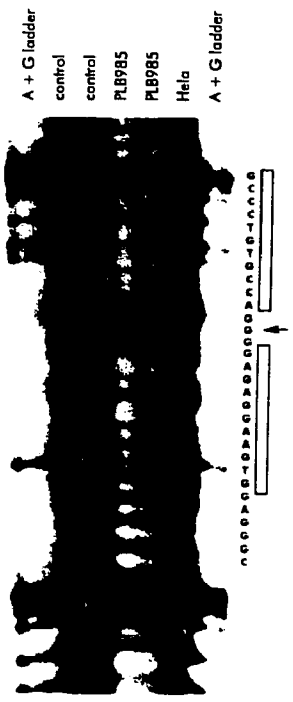


Fig. 7 DNAse I footprinting analysis on region between +7 and -196 on both strands. The results are summarized in this figure. The dark lines above or below the sequences indicate regions of complete protection whereas the lighter lines indicate regions of weaker protection. The protection on the anti-sense strand are indicated by lines drawn above the sequences and the protection on the sense strand are indicated by lines drawn below the sequences. Hypersensitive sites are indicated by arrows. The various putative binding sites are indicated by boxes over the sequences. Complete protection was observed over the 30 bp element, TATA box and the CCAAT box at -153 whereas Sp1 and c-myb showed no protection.

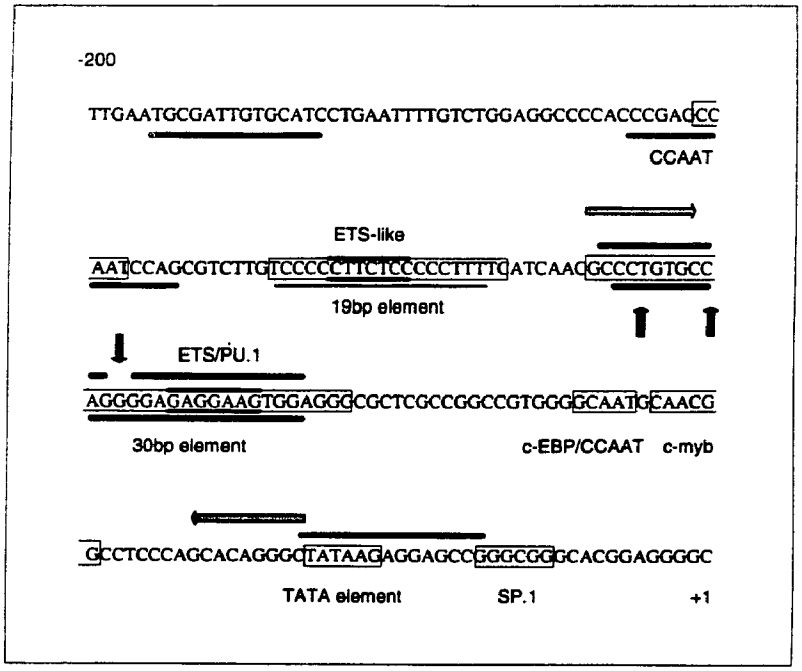

-200
TTGAATGCGATTGTGCATCCTGAATTTTGTCTGGAGGCCCCACCCGACCC
CCAAT
ETS-like
AATCCAGCGTCTTGTCCCCCTTCTCCCCCTTTTCATCAACGCCCTGTGCC
19bp element
ETS/PU.1
AGGGGAGAGGAAGTGGAGGGCGCTCGCCGGCCGTGGGGCAATGCAACG
30bp element
c-EBP/CCAAT
c-myb
GCCTCCCAGCACAGGGCTATAAGAGGAGCCGGGCGGGCACGGAGGGGC
TATA element
SP.1
+1

Fig. 8. South-western blot using the 30 bp element. Fifty micrograms of nuclear extracts from PLB-985 (lane 1), HL-60 (lane 3) and Hep G2 (lane 4) were separated on a 10% SDS-PAGE, transferred onto filters and probed with labeled duplex 30 bp element in the presence of 100ng/μl of poly (dI-dC). The various myeloid-specific proteins of 30, 38, 55-60 and 80 kDa binding to the 30 bp probe are indicated by arrows. A 42 kDa protein, present in all tissues examined, binds to the 30 bp element and is indicated by an unfilled arrow. The 30 and 80 kDa bands were of significantly lower intensity and appeared enhanced when lower concentrations of poly(dIdC) were used in the binding solution. The molecular weight markers are indicated to the right of the gel.

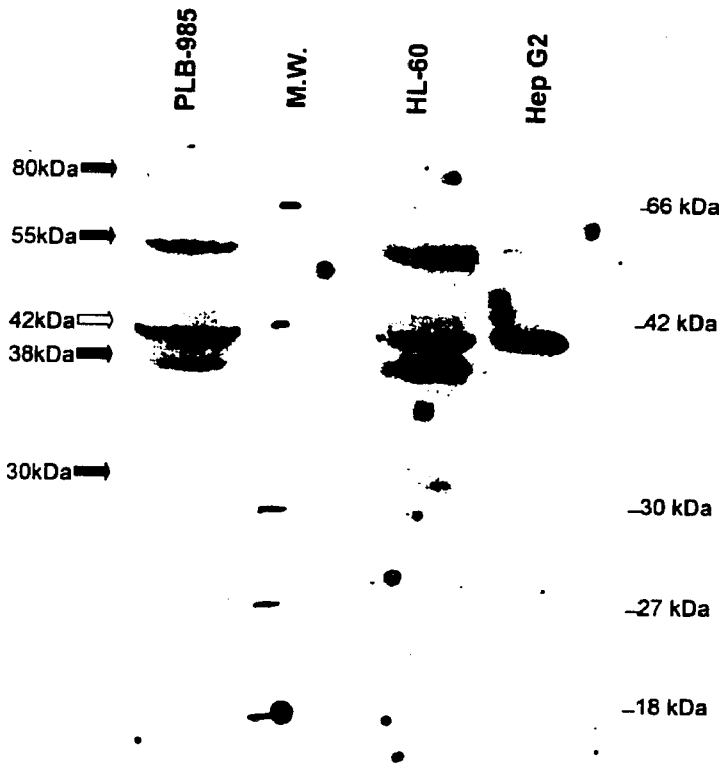

PLB-985
M.W.
HL-60
Hep G2
80kDa
55kDa
42kDa
38kDa
30kDa
66 kDa
42 kDa
30 kDa
27 kDa
18 kDa

Fig. 9. South-western blot using control GA rich probe. Fifty micrograms of nuclear extracts from HeLa (lane 1), PLB-985 (lane 2) and HL-60 (lane 3) were separated and probed as in Fig. 8 with the following modifications: 10ng/µl of poly (dI-dC) and G+A rich probe not capable of binding any myeloid specific proteins in EMSA assays were used to bind the proteins in the nuclear extracts. Two proteins of 42 and 80-90 kDa bound to this probe with high intensity and other proteins bound to this probe with low intensity.

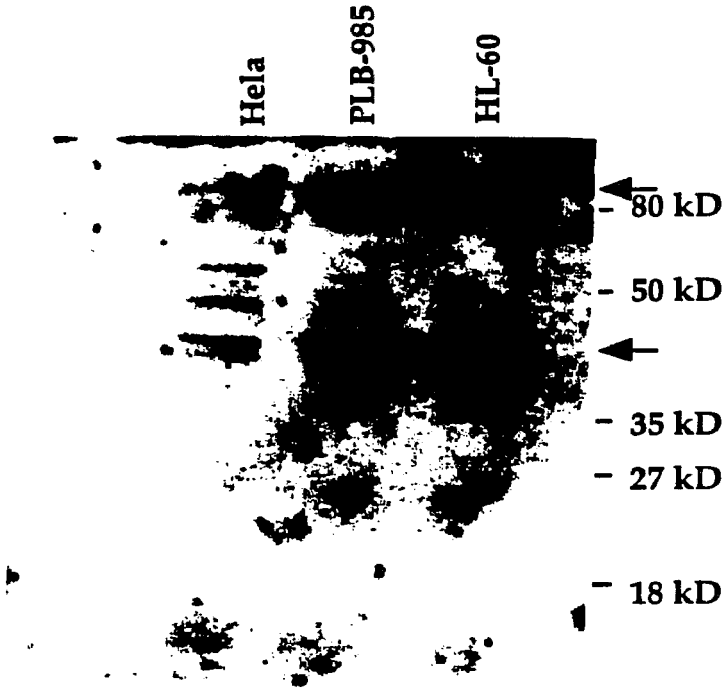
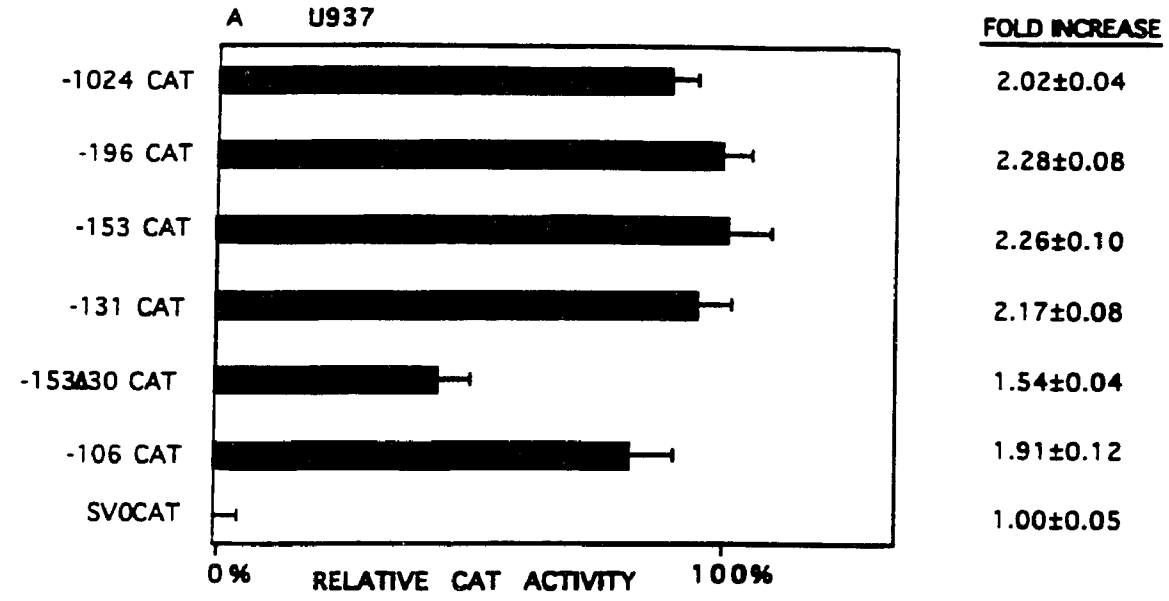
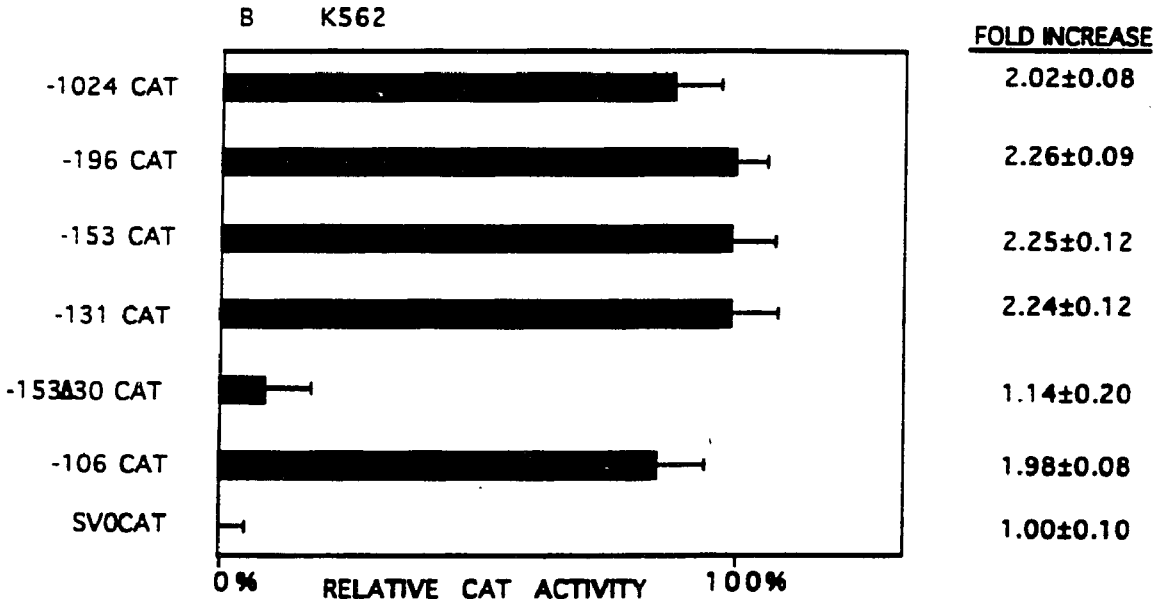

Hela
PLB-985
HL-60
80 kD
50 kD
35 kD
27 kD
18 kD

Fig. 10. HNE promoter deletion analysis in U 937 cells. A) Twenty micrograms of various reporter constructs are transfected along with 0.5 mg of pCMV-Luciferase internal control plasmid. After 48 hours, the cells are lysed and equal amounts of proteins used to perform the CAT assays. The amount of CAT in different transfections were estimated by liquid scintillation counting of acetylated chloramphenicol. All results have been corrected for transfection efficiency using luciferase as an internal control and repeated at least 3 independent times. Some of the transfections were repeated over 10 times for the sake of accuracy due to the low level of CAT activity seen in these experiments. The error bars indicate the actual range of measurements of corrected CAT activity seen in different reporter constructs. The graph displays expression of various constructs as a percentage of maximal expression seen with -153 CAT construct. The value of promoterless SV0CAT has been reduced to 0%. The corrected fold increases of various promoter constructs over promoterless control plasmid are also tabulated.
B) HNE promoter deletion analysis in K 562 cells. The methods and results are similar to those described for U 937 cells. C) HNE promoter deletion analysis in HeLa cells. No significant reporter activity was observed when promoter constructs were transfected into HeLa cells according to procedures mentioned above. The activity of the -153 CAT construct was lower or equal to the activity seen in promoterless SV0CAT.


A U937
-1024 CAT
-196 CAT
-153 CAT
-131 CAT
-153Δ30 CAT
-106 CAT
SV0CAT
0%
RELATIVE CAT ACTIVITY
100%
FOLD INCREASE
2.02±0.04
2.28±0.08
2.26±0.10
2.17±0.08
1.54±0.04
1.91±0.12
1.00±0.05


B K562
-1024 CAT
-196 CAT
-153 CAT
-131 CAT
-153Δ30 CAT
-106 CAT
SV0CAT
0%
RELATIVE CAT ACTIVITY
100%
FOLD INCREASE
2.02±0.08
2.26±0.09
2.25±0.12
2.24±0.12
1.14±0.20
1.98±0.08
1.00±0.10

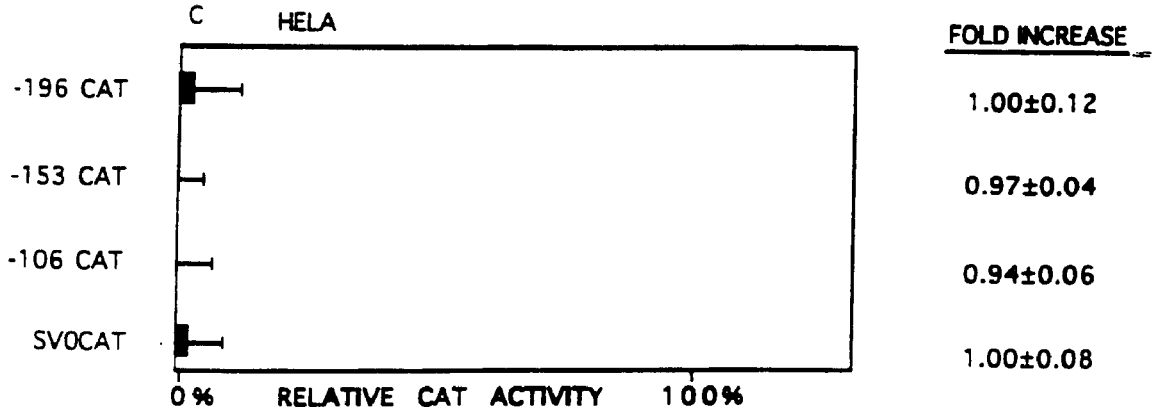
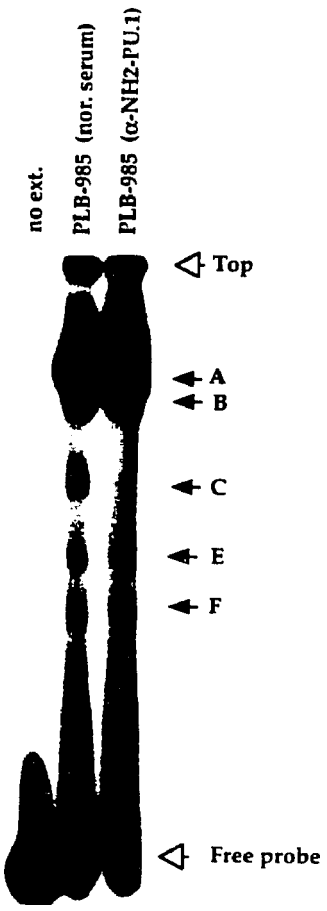

C HELA
-196 CAT
-153 CAT
-106 CAT
SV0CAT
0%
RELATIVE CAT ACTIVITY
100%
FOLD INCREASE
1.00±0.12
0.97±0.04
0.94±0.06
1.00±0.08

Fig. 11. PU.1 antiserum interacts with complex C in PLB-985 nuclear extracts. Five microliters of PU.1 antiserum were added to EMSA assays performed as in figure 1 and the reactions were run on a 6% non-denaturing gel. The complex C was abolished in EMSA with the 30 bp probe by the addition of the PU.1 antiserum (lane 3) but not by the addition of normal serum (lane 2).


no ext.
PLB-985 (nor. serum)
PLB-985 (α-NH2-PU.1)
Top
A
B
C
E
F
Free probe

PU.1 REGULATES THE EXPRESSION OF THE HUMAN NEUTROPHIL ELASTASE GENE.

by

SAMPATHKUMAR SRIKANTH AND THOMAS A. RADO

Submitted to

MOLECULAR AND CELLULAR BIOLOGY

1994

ABSTRACT

PU.1 is a transcription factor present in B cells and macrophages. Here, we report our studies on the role of PU.1 in myelopoiesis using Human Neutrophil Elastase (HNE) as a model. Human Neutrophil Elastase, a component of the primary granules of mature granulocytes, is a serine protease that is transcriptionally restricted to the late promyelocytic stage of granulocytic maturation. The first 200 bp of the HNE promoter directs myeloid specific expression of a reporter gene, and a 30 bp element within this region has been identified as the major determinant of myeloid specific expression. We now show that the B cell and macrophage specific transcription factor, PU.1, binds to the PU.1 consensus site within the 30 bp element to activate transcription. Substitution mutations within this recognition sequence results in the loss of PU.1 binding and in an 80-90% decrease in promoter activity in myeloid cells. We report the presence of multiple phosphorylated forms of PU.1 in the nuclear extracts of myeloid cells as demonstrated by phosphatase treatment and immunoblotting expreiments. The steady state level of PU.1 protein does not decline during differentiation of immature myeloid cells. Cotransfection of PU.1 and a reporter gene controlled by the HNE promoter into non-myeloid HeLa cells resulted in activation of reporter gene transcription. This data shows that PU.1 is an essential factor for myelopoiesis as characterized by its role in the expression of granulocyte and other macrophage specific genes.

INTRODUCTION

Human Neutrophil Elastase, a component of the azurophilic granules of granulocytes, is coordinately expressed in the promyelocytic stage of neutrophil maturation along with other genes including myeloperoxidase and the proteases myeloblastin, azurocidin, and cathepsin G (6, 9, 33). The human azurocidin, myeloblastin and neutrophil elastase genes have been reported to lie in a tight cluster of genes on the p terminus of chromosome 19, and another family, the cathepsin family of serine proteases, has also been reported to lie in a tight cluster of genes (13, 39). Previous

studies have shown that HNE mRNA expression is regulated at the transcriptional level and that sequences within 200 bp from the transcriptional start site in the 5' flanking region of HNE are sufficient to direct myeloid specific expression of the gene (32, 36). We have recently shown that a 30 bp element, with homology to elements in the promoters of other myeloid specific genes, is critical for directing reporter gene expression from the HNE promoter in hematopoietic cells (32). This element, containing a PU.1/Ets-binding consensus site, was also shown to bind multiprotein complexes present in the nuclear extracts of myeloid cells.

Spi-1/PU.1, a protooncogene, is a member of the Ets related family of transcription factors, which includes c-Ets-1 and -2, erg, elk-1 and 2, fli-1, GABPα, elf-1, SAP-1 and spi-B (16, 24). All members share homology in the 85 amino acid ETS DNA binding domain, and Spi-1/PU.1 is the most divergent member of this family (25). The expression of PU.1, previously thought to be B cell and macrophage specific, has now been reported in most hematopoietic lineages (7, 11, 25, 30). The pattern of expression of PU.1 in B cells, macrophages, basophils or mast cells, and in other myeloid cells suggests a functional role as a transcription factor in the tissue-specific expression of genes expressed in these lineages (3, 12, 17, 20, 28). Recently, it has been reported that PU.1 is upregulated during early myelopoiesis and addition of competitive binding sites for PU.1 to CD34+ bone marrow cells results in the inhibition of *in vitro* colony formation (34). Targeted disruption of the PU.1 gene in mice results in embryonic mortality and significant disruption of normal hematopoiesis (30). Specifically, mice homozygous for disrupted PU.1 have a virtual absence of multilineage progenitors for B and T lymphocytes, monocytes and granulocytes. The maturation, but not the generation of erythroid progenitors, is impaired, while the megakaryocyte lineage is unaffected (30). The absence of recognizable granulocyte progenitors in mice, which have a targeted disruption of PU.1, may reflect the role played by PU.1 in the transcription of granulocyte specific genes.

So far, the expression of several B cell, macrophage and mast cell lineage specific genes has been shown to be regulated by PU.1. Several B cell specific genes and enhancers including the immunoglobulin κ chain 3' enhancer, the immunoglobulin J chain promoter and the λ light chain 2-4 enhancer depend on PU.1 binding to its cognate site for their activity (4, 17, 22, 26, 31). Several macrophage specific genes including CD11b, CD18 and M-CSFR have also been shown to be functionally regulated by PU.1 (1, 3, 19, 20, 37). The PU.1 binding sites have been found in some viral promoters and enhancers, and may function in the tissue-specific expression of viral gene products in macrophages and B cells (2, 21). Recently PU.1, in combination with GATA-1 and 2, has been implicated in the expression of IL-4 from its intronic enhancer in mast cells (12).

In this study, we provide strong evidence for the crucial role of PU.1 in granulocytopoiesis. We define the nucleotide sequences in the 5' flanking region of the HNE gene that interacts with PU.1. We also show that PU.1 binds to the 5' flanking region of HNE and regulates the transcription of the gene. This demonstrates direct transcriptional regulation of HNE, a granulocyte specific gene, by PU.1. PU.1 binding sites are also found in the promoters of a number of granulocyte specific genes including proteinase 3, azurocidin, cathepsin G and myeloperoxidase, but the involvement of PU.1 in the regulation of these genes has not yet been demonstrated.

MATERIALS AND METHODS

Electrophoretic mobility shift assays (EMSA). Nuclear extracts were prepared from U 937, HeLa, K 562 and PLB-985 cells by the procedure described previously (32). The nuclear extracts were stored frozen at -70 ° C. The differentiation of PLB-985 and U 937 cells into monocytic cells was induced by the addition of 10^{-7} M Phorbol Myristic Acetate (PMA) (Sigma) to the culture media for 48 h. PLB-985 cells were differentiated into granulocytic lineage by the addition of 750μM dibutryl-cyclic AMP to the culture media for 48 h. The probes used for the EMSA were synthesized on an oligonucleotide

synthesizer. The sequences of the 30 bp oligonucleotide duplexes used in these studies are as follows: The 30 bp probe, representing the wild type promoter sequence, is 5' GCC CTG TGC CAG GGG AGA GGA AGT GGA GGG 3', the 30P probe in which the PU.1 core recognition site is mutated is 5' GCC CTG TGC CAG GGG ACG CCA AGT GGA GGG 3' and the 30M probe containing mutations in the conserved CCTGTGCC motif is 5' GCC AGT CAG GAG GGG AGA GGA AGT GGA GGG 3'. In order to create oligonucleotide duplexes, complementary oligonucleotides were annealed, gel purified and end labeled with ^{32}P γATP and T4 polynucleotide kinase. Twenty micrograms of nuclear extracts from different cells were added to the binding buffer containing 10mM Tris HCl (pH 7.5), 50mM KCl, 5mM $MgCl_2$, 1mM DTT, 1mM EDTA, 12.5% glycerol and 0.1% Triton X-100 with 3 μg of poly(dI-dC). The reaction was preincubated for 20 min. at 4°C. Then, 1 ng of labeled probe was added and incubation continued for another 20 min. Cold competition experiments were performed by adding 100-fold molar excess of unlabeled duplex oligonucleotides after the addition of labeled probe. Supershifts were performed by adding 7μl of PU.1 antiserum to the binding reaction prior to the addition of the probe. Three different anti-PU.1 antibodies were used: an amino terminus peptide antibody-1297 (a kind gift from Dr. R. Maki), a carboxy terminus peptide antibody (Santa Cruz Biotech, California) and an antibody directed against full length murine PU.1(9794) (a kind gift from Dr. D. Kabat). The reaction was then loaded onto a 6% or a 10% high ionic strength non-denaturing polyacrylamide gel containing 0.5 X TBE with 2% glycerol. The products were resolved and visualized by autoradiography.

DSP cross-linking. Protein cross-linking was performed in the presence of 0.5 mM Dithio succinylamide propionate (DSP) added to the binding reactions. For these experiments, the binding buffer used for EMSA contained 10mM HEPES instead of 10mM Tris HCl to avoid quenching of the cross linking reagent. After incubation at room temperature for 20 min., the reactions were directly loaded on a non-denaturing gel

as previously described. One microliter of 100mM DTT was added to the cross-linking reactions to reverse the effects of DSP. After incubation at 37 ^{o}C for 20 min., the samples were analyzed as described above.

Phosphatase treatment of EMSA reactions. Nuclear extracts were dephosphorylated by the addition of 2U of potato acid phosphatase (PAP) (Sigma) to the binding reactions. The reactions were incubated at 30 ^{o}C for 20 min. in phosphatase buffer containing 20mM HEPES (pH 7.9), 100 mM KCl, 0.2mM EDTA and 0.5mM DTT (23). The conditions were empirically determined by their ability to cause maximal dephosphorylation of nuclear extracts as determined by EMSA. Control nuclear extracts were mock treated in an identical manner in the absence of PAP.

Affinity column isolation of binding proteins. A pentameric clone of the 30 bp element was end-labeled with 14-biotin dATP and immobilized on streptavidin-agarose beads. One hundred micrograms of PLB-985 nuclear extract and poly (dI-dC) at a concentration of 0.1μg/μl were added to the immobilized probe in EMSA binding buffer and the binding reaction was allowed to continue for 2 h at 4 ^{o}C. The beads were centrifuged, washed six times in excess binding buffer and the bound proteins eluted from the probe by boiling. The eluted proteins were analyzed by SDS-PAGE and silver staining.

In vitro synthesis of PU.1. PU.1 was synthesized in bacteria using the pET-PU.1 plasmid bacterial expression system (a kind gift from Dr. D. Kabat). Briefly, the pET-PU.1 plasmid was transformed into BL21(pLysE) bacteria and protein production induced with IPTG () (Novagen). The bacterial extract was lysed by sonication and PU.1 protein production determined by silver-staining of SDS-PAGE. PU.1 was synthesized *in vitro* using rabbit reticulocyte lysate transcription/translation system (Promega). Briefly, murine and human PU.1 cDNA (kind gifts from Dr. R. Maki and Dr. R. Hromas) were transcribed *in vitro* with T_3 polymerase. The transcription products were translated in

the presence of ^{35}S methionine and the products analyzed by SDS-PAGE. The product of a parellel reaction using nonradioactive methionine was employed in EMSA and other experiments.

South-Western blot. Fifty micrograms of nuclear extract from differentiated and undifferentiated myeloid cells as well as 20 µl of PU.1 synthesized in reticulocyte-lysate were resolved on a 10% SDS-PAGE denaturing gel. The gel was run at 4 °C overnight at 10mA and then equilibrated in transfer buffer containing 25mM Tris, 192mM Glycine and 15% Methanol. The proteins were electroblotted onto Immobilon-P filters (Millipore) at 10 V/cm for 3 h. The proteins were denatured in 6M Guanidine hydrochloride solution in TNE-50 (10mM Tris HCl, 50mM NaCl, 1mM EDTA and 1mM DTT) for 5 minutes at room temperature. The filters were renatured by 2-fold dilution of the guanidine hydrochloride with TNE-50 over 30 min. The filters were blocked with 5% non-fat dry milk in TNE-50 for 1 h at room temperature and washed in the same buffer. The filters were bound in TNE-50 containing 10 ng/ µl of poly(dI-dC) and 200 ng of purified labeled 30 bp probe in 3 ml of binding solution for 1 h.at room temperature. The filters were washed in three changes of TNE-50, dried and autoradiographed.

Western blot. Thirty micrograms of nuclear proteins from differentiated and undifferentiated U 937 and PLB-985 cells were separated on a 10% SDS-PAGE. The proteins were electroblotted onto Immobilon-P (Millipore) filters as previously described and blocked with 5% non-fat dry milk in PBS containing 0.2% Tween-20 (PBS-T). Primary antiserum PU.1-9794, raised against murine full-length PU.1, was added at 1:1000 dilution in PBS-T, and incubated for 1 h at 4 °C. The primary antibody was washed in three changes of PBS-T and biotinylated protein A was added to the membranes at 1:1000 dilution. After 30 min at room temperature, the membranes were washed in three changes of PBS-T and bound with 1:10,000 dilution of streptavidin-alkaline phosphatase in PBS-T. The excess streptavidin was removed with three washes

in PBS-T and the color developed using Nitro-Blue-Tetrozolium (NBT) and Bromo-Chloro-Indolyl-Phosphate (BCIP). The reaction was stopped by washing in excess water.

Far-western Blot. Twenty micrograms of nuclear extracts were separated on SDS-PAGE and transferred onto Immobilon-P filters as described. The filters were blocked with 5% non-fat dry milk in PBS-T and washed in three changes of buffer at 4°C. One times 10^7 cpm of ^{35}S methionine-labeled human PU.1 synthesized in rabbit reticulocyte-lysate was used to probe the membranes in the presence of 2 mg/ml of BSA in binding buffer containing TNE-50. After incubation at 4 °C overnight, the filters were washed in four changes of TNE-50 buffer, dried and autoradiographed. As control, rabbit reticulocyte-lysate containing ^{35}S-methionine and all ingredients except T_3 polymerase was used to probe the same blots under identical conditions.

Transfections. The deletion and substitution mutations were created by PCR, verified by sequencing and subcloned into the pUMSCAT vector. Twenty micrograms of reporter test plasmid was precipitated in ethanol along with 0.5 μg of pCMV-Luciferase construct used as an internal control for transfection efficiency. Two times 10^7 U 937, K 562 or HeLa cells in logarithmic growth phase were harvested and resuspended in 700 μl of serum-free RPMI-1640 media. The differentiation of U 937 cells with 10^{-7} M PMA was carried out after transfection and the cells were harvested after 40 h. Plasmids were resuspended in 100 μl of serum-free RPMI-1640 media and added to electroporation cuvettes (BioRad) containing the cells. The cuvettes were placed on ice for 5 min. and then electroporated at 300 V and 960 μF (Gene pulser, BioRad). Following electroporation, the cells were placed on ice for 15 min. and transferred to flasks with RPMI-1640 media containing 20% fetal calf serum. After 48 h, the cells were harvested by centrifugation and resuspended in 400 μl of 1X reporter lysis buffer (Promega). The cells were lysed and the protein content in the cell-lysates assayed by BCA protein assays (Pierce). Equal amounts of protein (usually 200 μg) were used to perform Chloramphenical Acetyl Transferase (CAT) assays as per manufacturer's instruction

(Promega). The amount of CAT in the lysates was determined by liquid scintillation counting (Promega). The CAT reporter activity was corrected for transfection efficiency by standardization to luciferase levels determined on the same lysates (Promega). At least three independent transfections in duplicate were used to determine the reporter activity of different constructs.

Transactivation experiments were carried out using various amounts of activator plasmids pECE, pECE-PU.1 and pECE-c-Ets-2 (gifts from Dr. R. Maki) and 20 μg of reporter plasmids pUMS(-106)CAT and pUMS(-106P)CAT. Half a microgram of pCMV-Luciferase plasmid was used as internal control to normalize for equal transfection efficiency. After 40 h, the cells were lysed and CAT activity measured by liquid scintillation counting.

RESULTS

Mutation of the PU.1 binding site abolishes factor-binding to the 30 bp element . Our previous studies have shown that the 30 bp element binds at least six complexes in the presence of myeloid nuclear extracts (32). In order to characterize the proteins responsible for these complexes, we determined the effect of substitution mutations in the 30 bp element on its ability to bind proteins in nuclear extracts. Mutation of the putative PU.1 binding site from 5' GAGGAA 3' to 5' CGCCAA 3' (Oligonucleotide 30P or P) failed to form the major complexes (C to F) observed with wild-type 30 bp probe in PLB-985 nuclear extracts (Fig. 12, lane P). Complex A is not affected by this mutation. In contrast, substitution mutation of the conserved sequence between -103 and -97 from 5' CCTGTGCC 3' to 5' CAGTCAGG 3' (Oligonucleotide 30M or M) produced gel shift complexes A to F indistinguishable from those observed with the wild type probe (Fig. 12, lanes M) indicating that this site is not critical for the binding of the complexes. In cold competition studies using these mutant probes, 30P did not compete with the wild type probe for binding to the complexes A to F, while mutant probe 30M effectively competed away complexes C to F (Fig. 13). We have shown that complex A has a higher

affinity for DNA and hence requires more competitor for effective competition (32). These results indicate that the PU.1 binding site is critical for the formation of complexes C to F on the 30 bp element.

Antibodies to PU.1 interact with EMSA complexes formed with myeloid extracts . As shown above, the PU.1 binding site within the 30 bp element is essential for the formation of complexes C to F with the 30 bp probe. There are significant differences in the mobility shifts observed in U 937 or K 562 extracts and promyelocytic PLB-985 extracts (32). Only complexes A and C are observed in the presence of nuclear extracts from U 937 and K 562 cells, whereas EMSA performed with PLB-985 nuclear extracts displayed, in addition, complexes D to F. We investigated the nature of these complexes by performing EMSA in the presence of antibodies against PU.1. We used two different antibodies, antiserum against an amino terminus peptide of murine PU.1 and antiserum against full length murine PU.1, to investigate the complexes in PLB-985 nuclear extracts. As noted in Fig. 14, addition of normal serum does not affect any of the complexes but the addition of an antibody to amino-terminus of PU.1 specifically supershifts complex C and abolishes its binding (Fig. 14, lane 2) and addition of antiserum against whole PU.1 supershifts and hence abolishes the binding of complexes C, D and E (Fig. 14, lane 1). The intensity of complex F was decreased but the binding of complex F was not fully abolished. The supershifted complexes appear in the high molecular weight region of the gel around complex A. This indicates that all of the complexes C to F are recognized by PU.1 and may represent PU.1 related complexes.

Fig. 15 shows the results obtained with antibody against a peptide located in the DNA binding domain of PU.1. Controls for non-specific binding included normal serum and an antibody against the closely related proteins, Ets-1 and Ets-2. As shown in Fig. 15 (lanes 4, 7 and 10), the PU.1 antibody competes for the binding of complexes C in U 937 or K 562 extracts and for complexes C to F in PLB-985 extracts. Complex C, seen in all myeloid cells including PLB-985, U 937 and K 562 cells, is thus clearly immunoreactive

to PU.1 antibodies and represents the complex formed by PU.1 binding to the 30 bp element. Addition of excess anti-PU.1 antibodies completely competes the shifts but did not display any supershifted complexes (data not shown). Anti-c-Ets-1 and c-Ets-2 antibodies as well as normal serum do not interact or compete for binding of any of the complexes. The observation that the binding of complex F is abolished by the antibody to carboxy terminus peptide of PU.1 and by mutation of the PU.1 binding site suggests that this complex may be proteolytically related to PU.1. However, complex F does not interact with antiserum against whole PU.1 and the reason for this complex not being recognized by the whole PU.1 antibody is not clear (Fig. 14 and 17). Although the antibody raised against murine PU.1 has been previously shown to cross-react with human PU.1, this cross-reaction may be affected by post-translational modifications that occur to human PU.1 and could be responsible for some of the complexes not being recognized. Murine and human PU.1 have 100% homology in the DNA binding domain but differ considerably in the amino terminus. This may explain why the antibodies directed against the carboxy terminus peptide of PU.1 reacts with all the PU.1 related complexes seen in PLB-985 nuclear extracts. These studies also show that complexes C, D and E observed in PLB-985 extracts are PU.1-related. The proteins responsible for complexes A are not clearly defined by these studies and it cannot be ruled out that the high molecular weight complex A may represent the product of an interaction between PU.1 and other nuclear factors.

In vitro synthesized PU.1 binds to the 30 bp element . We synthesized murine and human PU.1 protein in rabbit reticulocyte lysate to determine the pattern of complexes formed when authentic murine and human PU.1 binds to the 30 bp element. Antisense human and murine PU.1, synthesized under identical conditions, were used as controls for non-specific interactions in EMSA. As shown in Fig. 16, two shifted complexes were observed when both murine and human authentic PU.1 bound wild type 30 bp oligonucleotide. These complexes comigrate with bands C and E obtained with PLB-985

nuclear extracts (Fig. 16, lanes 2, 3 and 6). Since reticulocyte lysates are rich in protein kinases, including casein kinase II, which are known to phosphorylate PU.1, complex C may correspond to a phosphorylated form of PU.1. The complex in reticulocyte lysates that comigrates with complex E present in PLB-985 nuclear extracts and may be due to binding of an internally initiated translation product (22). No differences were observed between the shifts produced by human and murine PU.1 proteins, and in all cases, the antisense PU.1 translation products did not bind to the probe (Fig. 16, lanes 4 and 5). Complexes A, B, D and F observed in EMSA with PLB-985 nuclear extracts were not observed in the presence of reticulocyte lysate synthesized PU.1.

Dephosphorylation of the binding reaction with phosphatase alters EMSA complexes .

PU.1 contains several serine and threonine residues in the amino terminus activation domain as a part of the region rich in proline, glutamic acid, serine and threonine residues (PEST region), and these can serve as substrates for a variety of cellular kinases. The results described above, with *in vitro* synthesized PU.1, suggest that some of the complexes observed with nuclear extracts may represent binding by differentially phosphorylated forms of PU.1. In order to test this hypothesis, the effect of dephosphorylation on the mobility of the EMSA complexes observed in PLB-985 nuclear extracts was studied by subjecting the binding reaction to acid phosphatase. As shown in Fig. 17, treatment of nuclear extract from PLB-985 cells with PAP for 30 min reduces the number of complexes from four to three and results in the concomitant appearance of bands that comigrate with complexes D, E and F. Complex C was affected distinctly by dephosphorylation and was completely abolished. Further, phosphatase treated complexes, that comigrate with complexes D and E, were supershifted by an antibody against full length PU.1 protein and the intensity of complex F was clearly reduced by the addition of the ani-PU.1 antiserum (Fig. 17, lane 5). These results suggest that the

complex C on EMSA may represent a highly phosphorylated form of PU.1 and that complexes D, E and F, seen in EMSA reactions with PLB-985 nuclear extracts, reflect different degrees of dephosphorylation.

This model was tested by using bacterially synthesized PU.1 in EMSA to determine the pattern of shifts obtained with the unphosphorylated protein. These studies demonstrate that the 30 bp probe bound to bacterially synthesized PU.1 comigrates with complexes D, E and F generated by dephosphorylation of PLB-985 nuclear extracts (Fig. 18, lanes 2 and 4). Additional complexes, indicated by an asterisk, were observed in extracts from uninduced bacteria and bacteria induced to synthesize PU.1. No complexes that comigrate with complex C were observed with bacterially synthesized PU.1 (Fig. 18, lane 4). This finding lends evidence to the presence of differentially phosphorylated forms of PU.1 in PLB-985 extracts. Immunoblot analysis of bacterially synthesized PU.1 revealed only two proteins that are recognized by anti-PU.1 antibodies (see Fig. 20) and it is unclear why multiple bands were observed in EMSA with these extracts.

Expression studies using substitution mutants of PU.1 binding site . Our earlier mutational experiments using EMSA have shown that mutation of the PU.1 binding site results in the complete abolition of complexes C to F. To determine whether the ability of these complexes to bind the 30 bp probe has functional significance, we introduced these mutations into pHNE-106CAT and used these vectors to transfect myeloid and non-myeloid cells. U 937 promonocytic leukemic cells are myeloid cells which express PU.I and HNE (32). K 562 cells are early myeloid blast-like cells with the ability to express markers characteristic of different hematopoietic lineages in response to differentiation inducing agents. These cells express PU.1 but have not been observed to express HNE. U 937 and K 562 were used in this study as myeloid cell models because they express PU.1 and, unlike promyelocytic cell lines PLB-985 and HL-60, are readily transfected at acceptable efficiencies. The results presented in Fig. 19 show that mutation of the PU.1 binding site (-106PCAT) results in an 80-90% loss of promoter activity in U 937 and K

562 cells (Panel A, dark bars). The wild type promoter had no activity in HeLa cells (Panel C). In contrast, the mutation -106MCAT, which produced a pattern similar to wild-type probe in EMSA studies, retained 95% of the wild type promoter activity in U 937 cells (Panel A, dark bars). The activity of the wild-type and mutant promoters in K 562 cells is similar to that observed in the U 937 line (Panel B).

Since HNE mRNA is down-regulated when U 937 cells are induced to differentiate in response to phorbol esters, experiments were performed to determine if expression from the transfected HNE promoter was affected by the differentiation state of U 937 cells. We transfected -106 HNE CAT, -106MCAT and -106PCAT into U 937 cells and induced differentiation with 10-7M phorbol ester. After 40 h. in this medium, greater than 95% of the cells were adherent and displayed monocytic morphology. CAT activity was measured in these cells and found to be similar to that observed in undifferentiated U 937 cells (Fig. 19, Panel A light gray bars). This suggests that the level of PU.1 activity is not significantly altered by differentiation of U 937 cells. These experiments suggest that HNE promoter expression, conferred by the 30 bp element, displays specificity for cells that express PU.1, but does not fully account for the stage restricted specificity of the native HNE gene expression.

Differentiation of myelomonocytic and promyelocytic cells does not alter steady state PU.1 protein level . The expression studies described above suggest that PU.1 levels are not significantly affected by differentiation of U 937 cells. We examined the steady state levels of PU.1 protein directly by immunoblot and southwestern analysis performed on differentiating myelomonocytic U 937 and promyelocytic PLB-985 cells. Equal amounts of nuclear protein, confirmed by Coomassie staining and Bradford assays, were separated by a SDS-PAGE and transferred to membranes. The blots were probed with anti-full length PU.1 antiserum. The steady state amount of PU.1 protein did not change significantly when PLB-985 was induced to differentiate into granulocytes with cAMP or into monocytes with phorbol ester (Fig. 20, lanes 5, 8 and 9). The amounts of PU.1 protein also remained constant in U 937 cells after differentiation with PMA (data not

shown). Surprisingly, several protein bands ranging from 36 to 50 kDa were recognized in myeloid nuclear extracts by the anti-PU.1 antibody. This is consistent with the presence of differentially phosphorylated forms of PU.1, which have been reported to exist in nuclear extracts (29). These bands were observed in K 562 nuclear extract as well as in the lane containing *in vitro* synthesized PU.1 from the reticulocyte lysate system (Fig. 20, lanes 2, 3 and 4). Bacterially synthesized PU.1 (positive control against which the antiserum was raised) was recognized as a single 38 kDa band and no signal was seen in lysate of uninduced bacteria (Fig. 20, lanes 6 and 7). The 9794 anti-PU.1 antiserum has been reported to immunoprecipitate several proteins of 38-45 kDa all of which are related to PU.1 and which are thought to represent differential phosphorylated forms of the protein (29). An additional band of 80 kDa was observed by us in all extracts and this band could indicate the existence of a non-specific cross-reacting protein recognized by the antibody. Similar results were obtained when the blots were probed with antiserum against amino terminus and carboxy terminus peptides of PU.1 (data not shown).

The binding activity of PU.1 in nuclear extracts was examined by probing blots with the 30 bp element containing the PU.1 binding site. Reticulocyte-synthesized human PU.1 was used to identify the mobility of bands corresponding to PU.1. These studies showed that the binding activity of PU.1 in nuclear extracts does not change significantly after induction of differentiation in PLB-985 cells (Fig. 21, lanes 2, 3 and 4). *In vitro* synthesized PU.1 displayed binding activities of 37- 50 kDa in the presence of low amounts of non-specific competitor poly (dI-dC), whereas control reticulocyte lysate proteins did not bind any probe (Fig. 21, lanes 8 and 9). Undifferentiated and differentiated U 937 nuclear extracts displayed a similar binding pattern. Nuclear extracts from PLB-985 cells, induced to differentiate with cAMP, showed similar amounts of binding activity as seen in undifferentiated PLB-985 nuclear extracts whereas the binding activity in PLB-985 cells, induced to differentiate with PMA, showed a

relative decrease in binding activity (Fig. 21, lanes 2, 3 and 4). This is interesting since cAMP induces granulocytic differentiation in PLB-985 cells and PMA induces monocytic differentiation in these cells.

Authentic human PU.1, synthesized in rabbit reticulocyte lysate, migrated as four distinct bands of 27, 35, 37 and 46 kDa (Fig. 21, lane 7). It is difficult to correlate these bands with the two complexes seen in EMSA studies utilizing *in vitro* synthesized PU.1. These bands were also observed in nuclear extracts from the hematopoietic cell lines examined in these experiments and correspond to multiple phosphorylated forms of PU.1. In addition, the myeloid nuclear extracts examined contained proteins of 18-25 kDa, 55-60 kDa and 85-95 kDa. These bands were not observed in *in vitro* synthesized PU.1 lanes. The 18-25 kDa signals may represent proteolytic cleavage products since PU.1 contains a PEST domain distinct from the DNA binding domain (27). The identity of the 55-60 kDa and 85-95 kDa proteins is not known, and the significance of their binding to the PU.1 recognition site remains unclear.

A DNA affinity column, in which a pentamer of the 30 bp element was linked to a solid phase support, was used to achieve partial purification of nuclear proteins that bind to the 30 bp element. Such affinity purifications can result in 100-200-fold enrichment of DNA binding proteins. At least five different proteins of 18, 32, 36, 55 and 85 kDa were identified in PLB-985 nuclear extracts using this approach (Fig. 22, arrows). These proteins correspond to the major bands identified by south-western analysis using the 30 bp element and may play a functional role in its transcriptional activity. We can only speculate on the nature of the 55 and 85 kDa proteins seen in the south-western blots and in the DNA affinity column.

PU.1 interacts with other nuclear proteins . The results from EMSA, south-western and DNA affinity column analyses suggest that proteins other than PU.1 also bind to the 30 bp probe. The possibility that these proteins interact closely was investigated by exposure of the EMSA binding reaction to bifunctional chemical cross-linking reagents.

EMSA reaction mixture containing PLB-985 nuclear extract and the 30 bp probe was treated with various concentrations of dithio succinylamide propionate (DSP). This reagent cross-links proteins that are in close contact by means of a 12Å spacer arm. The cross-linking is reversed by addition of a thiol reagent such as dithiothreitol (DTT). As concentration of DSP was increased, complexes C to F normally seen in PLB-985 extracts acquired slower mobilities suggesting formation of large cross-linked complexes (Fig. 23, lanes 5 and 6). At the highest concentrations of DSP, all of the complexes A to F were cross-linked and the resulting multimer could not be resolved in a high percentage gel (Fig. 23, lanes 3 and 4). Addition of DTT to the reaction after cross-linking reversed the process and restored the initial pattern of shifted bands suggesting a specific protein-protein interaction process (Fig. 23, lane 2). The complexes indicated by unmarked open arrows were observed when EMSA is carried out in the presence of HEPES buffer and its significance remains unclear. The high molecular weight complex formed may arise from homomultimerization of PU.1. To evaluate this possibility, *in vitro* synthesized PU.1 was substituted for nuclear extracts in the binding reactions. As shown in Fig. 23, lanes 10, 11 and 12, addition of DSP does not lead to the formation of slow mobility complexes strongly suggesting that the protein-protein interactions observed in PLB-985 extracts actually reflect interaction between PU.1 and other nuclear proteins (lanes 3 to 6). It is interesting to note that complex A was not observed in EMSA carried out in the presence of reticulocyte lysate PU.1. This suggests that complex A arises from interaction between PU.1 and other protein(s) that bind the 30 bp probe.

In order to determine the size and the number of proteins present in the nuclear extracts that interact with PU.1, blots of SDS-PAGE separated nuclear extracts were probed with ^{35}S methionine labeled human PU.1 synthesized *in vitro* ("Far western analysis"). As shown in Fig. 24, numerous nuclear proteins are recognized by PU.1. Of particular interest are the bands representing proteins of 25-35 kDa and 105-150 kDa. These correspond to the molecular weights of TATA binding protein (TBP) and

retinoblastoma (Rb) protein, both of which have been reported to interact directly with the activation domain of PU.1 (10). No signal was detected in the lane containing reticulocyte lysate synthesized PU.1 indicating that PU.1 does not form homodimers or higher order multimers (Fig. 24, Lane 5). As a control for non-specific interactions, blots were probed with reticulocyte lysate containing ^{35}S methionine but no PU.1 mRNA (Fig. 24, lanes 1,2 and 3) and no signal was detected in these blots. The identity of the proteins that interact with PU.1 remains unclear and these interactions may have functional significance in the biological role of PU.1. Interestingly, no major differences in the proteins that interact with PU.1 were detected between myeloid and non-myeloid nuclear extracts in these studies. This is consistent with the observation that all the proteins known to interact with PU.1, with the exception of NF-EM5, are found in a wide variety of tissues.

Cotransfected PU.1 activates expression from the HNE promoter . Since several proteins, including Ets related proteins, can activate transcription from the PU.1 binding site, we performed experiments to determine whether transcription from the HNE promoter displays a specific requirement for PU.1. HeLa cells, which do not express PU.1, were cotransfected with the eukaryotic expression plasmids pECE-PU.1 or pECE-c-Ets-2 along with HNE-106 CAT construct, which includes the PU.1 binding site. In all of these experiments, pCMV-luciferase was used as an internal control to normalize results for transfection efficiency. As shown in Fig. 25, top panel, cotransfection of HeLa cells with HNE-106CAT and PU.1 expression plasmid resulted in a two-fold increase in reporter gene expression. In contrast, cotransfection with HNE-106CAT and the c-Ets-2 expression vector resulted in levels of CAT expression, which were not significantly different from those observed after cotransfection with HNE-106CAT constructs and pECE plasmid with no insert (Fig. 25, bottom panel). In parellel experiments, cells were transfected with the HNE-106PCAT vector that contains a mutated PU.1 binding site. These experiments show that activation of transcription requires an intact PU.1 binding

site. As shown in Fig. 25, top panel, pECE-PU.1 failed to activate transcription of the plasmid pHNE-106PCAT in HeLa cells. This construct displayed only 13% of the activity of the wild type HNE-106CAT construct when equal amounts of pECE-PU.1 were cotransfected (Fig. 25. top panel, open squares). As expected, the pECE plasmid without insert does not affect expression from either reporter construct (data not shown). The expression of PU.1 and c-Ets-2 does not affect the expression of luciferase from the CMV-luciferase internal control plasmid when assayed independently, indicating that the increased CAT expression observed in pECE-PU.1 cotransfections is not an artifact resulting from suppression of luciferase expression (data not shown). The level of activation observed after cotransfection of pECE-PU.1 and HNE-106CAT is equivalent to the level of activity observed with the native promoter in myeloid cells (Fig. 19). The modest levels of transactivation observed with the PU.1 binding site in the native promoter context was repeatable and similar levels of activation by PU.1 have been observed by others (22, 31).

DISCUSSION

The experiments described in this report provide strong evidence that PU.1 plays an important role in the regulation of transcription of HNE. *In vitro* synthesized PU.1 binds to a 30 bp element, derived from the HNE promoter, producing mobility shifted complexes that comigrate with some of the shifts observed with nuclear extracts from myeloid cells. Antibodies to PU.1 recognize and supershift complexes C to F found in nuclear extracts indicating that these complexes are PU.1 related (Fig. 14, 15). Mutation of the binding site for PU.1 in the HNE promoter, which results in the abolition of complexes C to F, (-106PCAT), resulted in an 80-90% loss of promoter activity in U 937 cells (Fig. 19). PU.1, but not c-Ets-2, when cotransfected with the HNE-106CAT vector into HeLa cells, resulted in transactivation of reporter gene expression, while mutation of the PU.1 binding site within the native promoter completely abolished the transactivation observed with PU.1 indicating specific interactions (Fig. 25).

A novel finding in these studies is that the steady state levels of PU.1 protein, examined by immunoblotting, remain constant during differentiation of PLB-985 and U 937 cells (Fig. 20-22) and that gene expression driven by the HNE promoter is not down regulated in U 937 cells that have been induced to differentiate (Fig. 19). Hromas *et al.* reported that PU.1 is expressed in granulocyte and monocyte precursors in normal bone marrow but is not present in the mature stages of granulocyte development (11). These apparently conflicting results may reflect dysregulation or incomplete differentiation of leukemia cells, and further investigation may shed light on the mechanisms responsible for stage restricted gene expression in the granulocyte lineage. The other possibility is that artificial induction of differentiation does not mimic the natural process of granulocyte and macrophage differentiation. For example, artificial induction of differentiation of HL-60 cells does not result in the upregulation of myeloid specific genes encoding for secondary granule components. This difference in PU.1 levels may reflect the results of such an abberant expression pattern seen in leukemic cells.

Recent evidence from the targeted disruption of PU.1 gene in mice suggests that PU.1 is critical for the development of the early hematopoietic system (30). Disruption of PU.1 results in prenatal mortality due to the elimination of precursors for granulocytes, macrophages, B and T cells (30). It has been shown that treatment of CD34+ hematopoietic progenitors with an oligonucleotide that contains the PU.1 recognition site, and therefore binds PU.1, results in the inhibition of *in vitro* hematopoiesis (34). While it has been shown that PU.1 is involved in the regulation of several B cell and macrophage specific genes, HNE is the first granulocyte specific gene shown to require PU.1 for its expression. Our results are consistent with the finding that disruption of PU.1 causes the developmental abnormalities in the granulocyte compartment of homozygous spi-1-/- mice (30).

Three complexes (D to F) were observed in the EMSA using PLB-985 nuclear extracts but not in U 937 and K 562 nuclear extracts. Our investigations revealed that

these complexes (D to F) increase in intensity with treatment with phosphatase, comigrate with unphosphorylated PU.1 and are immunoreactive to PU.1 antibodies. We have consistently observed these shifts only in promyelocytic nuclear extracts and the pattern of shifts does not alter between different preparations. We conclude that these EMSA complexes arise from differential phosphorylation of PU.1. Although PU.1 is known to be phosphorylated at different residues, no differences in phosphorylation states of PU.1 between U 937 and PLB-985 cells have been reported so far. The exact functional significance of the differentially phosphorylated forms of PU.1 and their effects on gene expression and protein interactions have not yet been elucidated. However, post-translational modification has been identified for the c-Ets-1 protein, and a correlation exists between the occurrence of highly phosphorylated forms of c-Ets-1 and cell cycle associated processes (5, 14). The relationship between functional activity of PU.1 and the cell cycle has not yet been studied, and phosphorylation may be a mechanism by which the function of PU.1 is regulated.

We also observe, in our EMSA studies, that a complex with very slow mobility (Complex A) is present only in nuclear extracts from myeloid cells and may represent the result of multi-protein interactions. The cross-linking studies and far-western analyses described in this report demonstrate that PU.1 interacts with other nuclear proteins in myeloid cells. Similar interactions have been reported for other Ets related proteins (15, 17, 26, 35) and several recent studies report such interactions for PU.1. PU.1 is known to interact with the B cell specific cofactors NF-EM5 and JB-1 (4, 23, 28). The activation domain of PU.1 interacts with TBP and with the cell-cycle related retinoblastoma protein (Rb) (10). It has been suggested that the binding of PU.1 to low affinity sites may be enhanced by the formation of PU.1-cofactor complexes (31). Zhang *et al.* have proposed that PU.1 acts by recruiting nuclear factors to the transcriptional complex in TATA-less promoters (37). Our results suggest that while PU.1 alone is a weak activator of transcription, its ability to form multi-protein complexes may serve to regulate high level

of in vivo expression of lineage specific genes. The specific pattern of expression of different lineage specific genes could be defined by complex combinatorial interactions between general and tissue specific factors. Our far-western analysis failed to identify any unique protein interaction between PU.1 and other proteins in nuclear extracts from myeloid cells. This suggests that PU.1 can transactivate the HNE promoter in non-myeloid cells because the additional factors that are required for functional activity of PU.1 are present in all tissues. Phosphorylation of specific residues in PU.1 may serve as one of the mechanisms by which these protein-protein interactions are regulated.

Very recently, PEBP2, a protooncogene, has been implicated in the regulation of murine myeloperoxidase and neutrophil elastase gene (18, 32). Although considerable homology exists between the human and murine elastase promoters, the consensus PEBP2 consensus binding site is clearly absent in the human elastase promoter and PEBP2 could not cotransactivate murine elastase promoter in non-myeloid cells (18). In contrast, the PU.1 consensus binding site is conserved between the human and murine elastase promoters, and PU.1 binding sites can also be found in the promoters of other granulocyte specific genes including azurocidin, proteinase 3, cathepsin G and myeloperoxidase (32). We clearly show in these studies that PU.1 is the main determinant of expression from the HNE promoter by cotransactivation experiments in non-myeloid cells. It is likely that factors like c-myb and PEBP2 are not expressed in HeLa cells and are apparently not critical for the myeloid specific transcriptional activity of the HNE promoter. The PEBP2 results may reflect differences in the regulation between the murine and the human elastase expression. Although factors like c-myb have been shown to be functionally important in the expression of myeloid specific genes in the mouse, no functional targets have been described for this protooncogene in the promoters of human myeloid specific genes. Our future studies are aimed at elucidating

the role of PU.1 in the orchestrated regulation of genes expressed during the promyelocyte stages of myeloid differentiation and in particular, of the genes in the elastase cluster.

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Fig. 12. Mutation of the PU.1 binding site affects binding of EMSA complexes. The wild type PU.1 binding site was mutated from 5' GAGGAA 3' to 5' CGCCAA 3' (denoted as P) within the context of the wild type 30 bp element. The mutation of the M element is described in the materials and methods section. The wild type, P and the M probe were used as substrates in EMSA reactions containing PLB-985 nuclear extracts. The products were resolved on 10% non-denaturing gel and visualized by autoradiography. Note that the mutation of the PU.1 binding site abolishes binding of complexes C to F (lane 2) whereas mutation of the M region did not affect binding of the complexes (lane 4). Shifts obtained with wild type probe are shown in lane 5. Complex A was not affected by the mutation of the PU.1 binding site.

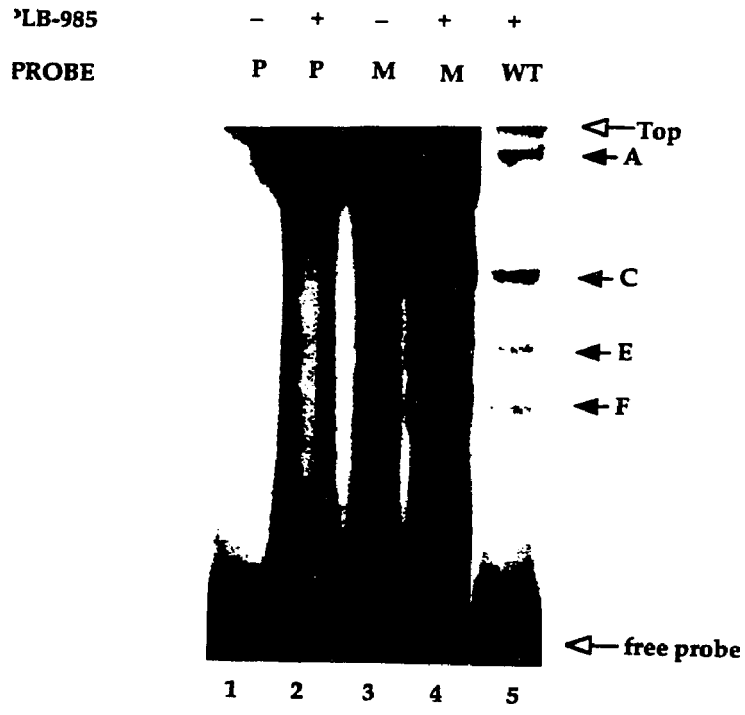

'LB-985
– + – + +
PROBE
P P M M WT
Top
A
C
E
F
free probe
1 2 3 4 5

Fig. 13. Probe P failed to compete any of the EMSA complexes. 100-fold excess of the unlabeled duplex oligonucleotides were added to binding reactions prior to the addition of the probe. EMSA reactions were carried out as described above. Probe P failed to compete any of the complexes (lane 4) whereas the wild type probe and probe M effectively competed away all the complexes (lanes 3 and 5). The shifts obtained with wild type 30 bp probe are shown in lane 2.

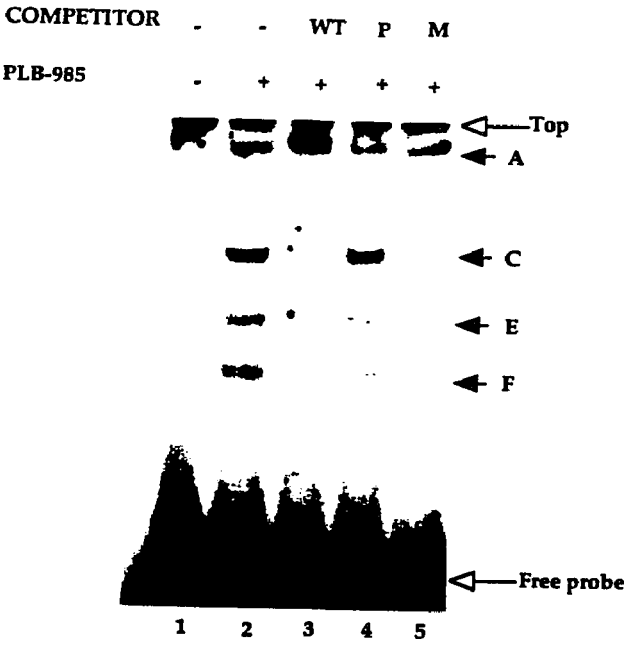

COMPETITOR
- - WT P M
PLB-985
- + + + +
Top
A
C
E
F
Free probe
1 2 3 4 5

Fig. 14. Antibodies against PU.1 supershift EMSA complexes. Two different antibodies against PU.1 were added to EMSA reactions containing PLB-985 nuclear extracts. EMSA reactions were performed as described above. Lane 1 shows the results of addition of 9794 antiserum against whole PU.1 to the binding reactions. Complexes C, D and E react with the antiserum and are supershifted to a complex denoted as S. Lane 2 reveals the interaction between an antibody raised against an amino terminus peptide of murine PU.1 and the EMSA complexes. Complex C alone reacts with this antibody and forms the supershifted complex S. Normal serum does not interact with any of the shifted complexes (lane 3) and shows the normal gelshift pattern.

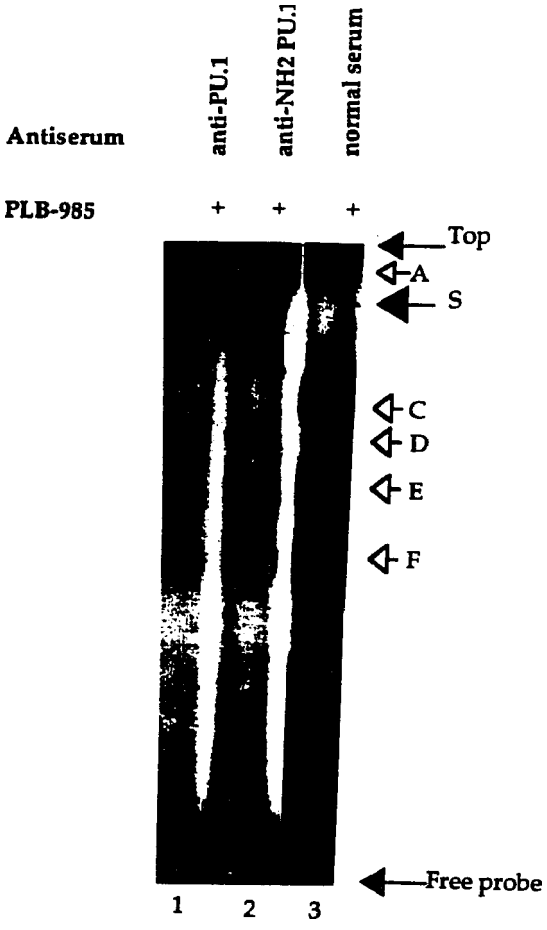

Antiserum
anti-PU.1
anti-NH2 PU.1
normal serum
PLB-985
+
+
+
Top
A
S
C
D
E
F
Free probe
1
2
3

Fig. 15. Effect of addition of antibodies to a conserved carboxy terminus peptide of PU.1. Addition of antibodies against an carboxy terminus peptide of PU.1 (Santa Cruz Biotech) to the EMSA reactions resulted in the competition of complexes C to F observed in myeloid cells (lanes 4, 7 and 10). Note that the K 562 and U 937 nuclear extracts do not give an identical pattern of shift compared to PLB-985 extracts, but complex C is competed away by the addition of the antibodies (lanes 7 and 10). Addition of normal serum (lanes 2, 5 and 8) and antibodies against c-Ets-1 (lanes 3, 6 and 9) does not affect the binding of any of the complexes.

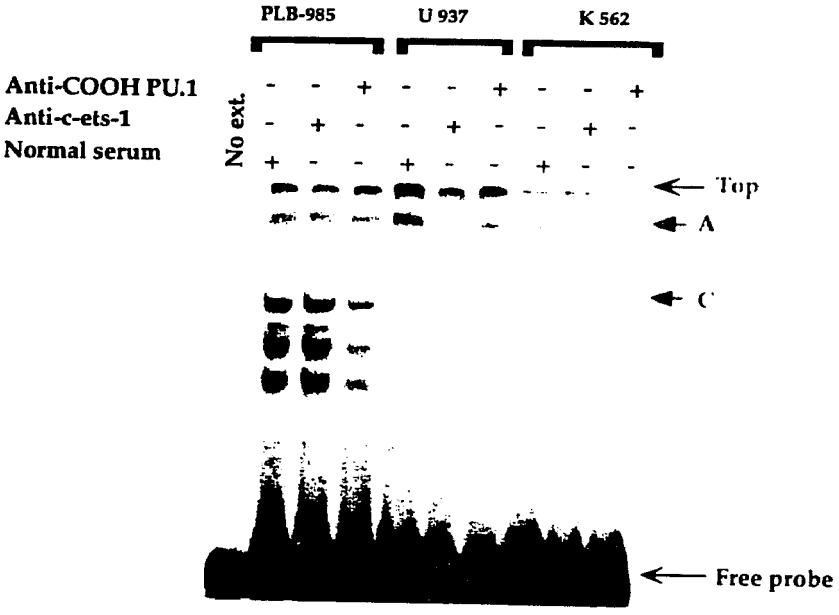

PLB-985
U 937
K 562
Anti-COOH PU.1
Anti-c-ets-1
Normal serum
No ext.
Top
A
C
Free probe

Fig. 16. In vitro synthesized PU.1 binds to the 30 bp element. Human and murine PU.1 were synthesized in the rabbit reticulocyte lysate system and used to bind the 30 bp element in EMSA reactions. Both human and murine PU.1 (lanes 3 and 7) specifically bound to the probe and these shifts comigrated with the shifts C and E observed in PLB-985 extracts (lane 2). No binding activities were observed with reticulocyte lysates programmed to synthesize antisense human and murine PU.1 (lanes 4 and 5). No complex corresponding to complex A was observed in the in vitro synthesized PU.1 reactions.

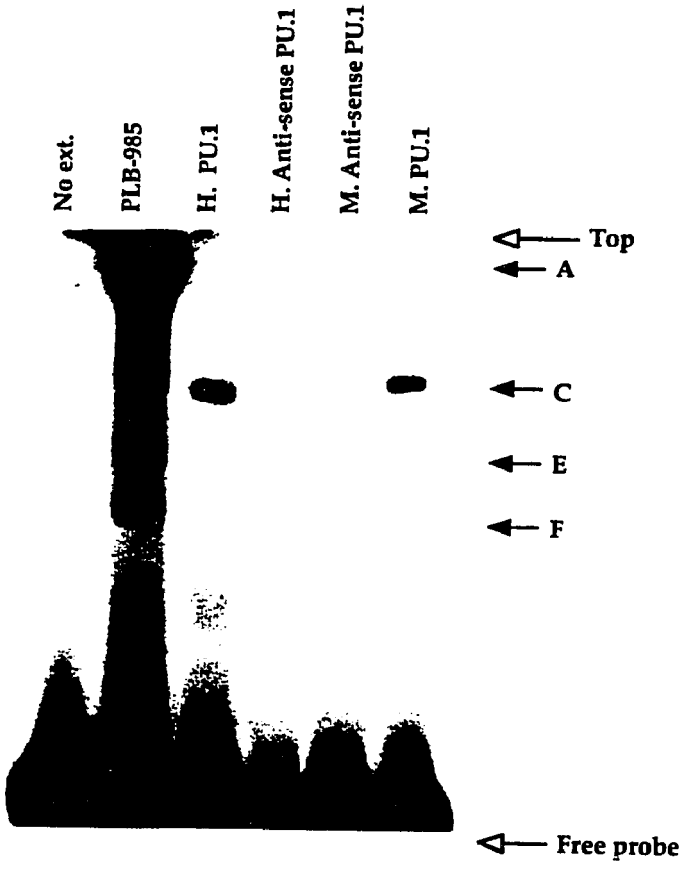

No ext.
PLB-985
H. PU.1
H. Anti-sense PU.1
M. Anti-sense PU.1
M. PU.1
Top
A
C
E
F
Free probe

Fig. 17. Phosphatase treatment of nuclear extracts affects binding of complex C. Potato Acid Phosphatase (PAP) was added to the binding reactions prior to the addition of the probe. The reactions were then incubated at 30°C for 30 minutes and subjected to EMSA reactions as above. PLB-985 nuclear extracts were mock treated in identical conditions without PAP to control for the effects of incubation. Lane 4 shows the effect of treatment of PLB-985 nuclear extracts with PAP. Complex C is completely abolished and the intensity of complexes D, E and F increases. Mock treated (lane 3) and normal PLB-985 nuclear extracts (lane 1) appear identical. Addition of 5µl of anti-PU.1 antibodies to the PAP-treated nuclear extracts resulted in the supershifting of complexes C, D and E suggesting that these complexes are indeed PU.1 related and not the result of artifacts associated with PAP treatment of nuclear extracts.

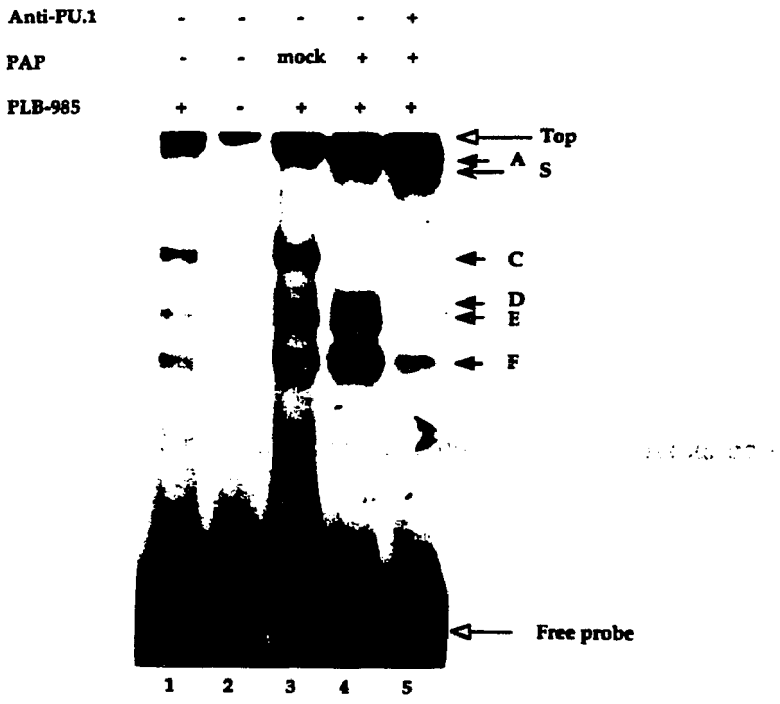

Anti-PU.1 - - - - +
PAP - - mock + +
PLB-985 + - + + +
Top
A
S
C
D
E
F
Free probe
1 2 3 4 5

Fig. 18. Bacterial PU.1 comigrates with some of the EMSA complexes. 2μl of bacterial extract containing PU.1 was added to EMSA reactions (lane 4) and the shift pattern determined. Uninduced bacteria to which no IPTG was added was also included in these reactions as controls (lane 3). Bacterially synthesized PU.1 produced three complexes that comigrates with complexes D, E and F found in PLB-985 nuclear extracts (lane 2). A non specific complex (indicated by *) was also observed in both uninduced and induced bacterial extracts. Note that complexes A and C are not present in bacterial extracts containing PU.1.

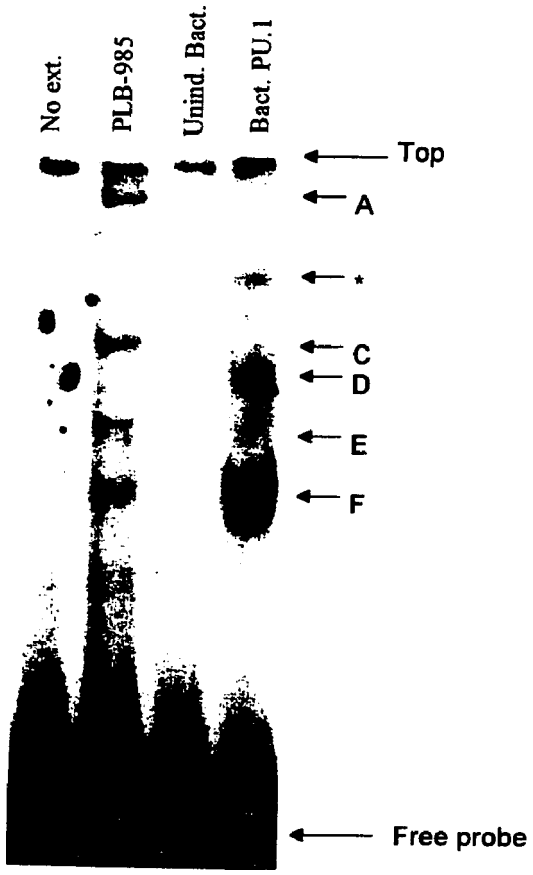

No ext.
PLB-985
Unind. Bact.
Bact. PU.1
Top
A
*
C
D
E
F
Free probe

Fig. 19. Mutation of PU.1 binding site markedly affects functional activity. Mutations in the region of the 30 bp sequence were introduced into the vector HNE-106CAT, transfected into U 937, K 562 and HeLa cells.

A. 20μg of -106CAT, -106PCAT and -106MCAT constructs were electroporated into 2 x 10^7 U 937 cells. After transfection, these cells were divided into two aliquots and one was treated with 10^{-7}M PMA. The cells were lysed and CAT activity assayed after 40 hours. The CAT activities were normalized with internal control luciferase levels and presented as a percentage of activity of the wild type HNE-106CAT vector. The activity in differentiated cells is represented by light shaded bars. Mutation of the PU.1 binding site (-106PCAT) results in 85-90% loss in promoter activity in both undifferentiated and differentiated U 937 cells (black and shaded bars). HNE-106MCAT displayed activities similar to those of the wild type promoter. Differentiation of U 937 cells (shaded bars) did not affect the activities of any of the constructs.

B. The activities of the various promoters were assayed in K 562 cells as described above except that no differentiation inducing agents were added after transfection. The activities of the -106CAT, -106PCAT and -106MCAT constructs were similar to the activities observed in U 937 cells.

C. The wild type -106CAT and the promoterless vector SVOCAT were transfected into HeLa cells and CAT activities determined as described above. The HNE promoter failed to express activity above that observed with the promoterless construct.

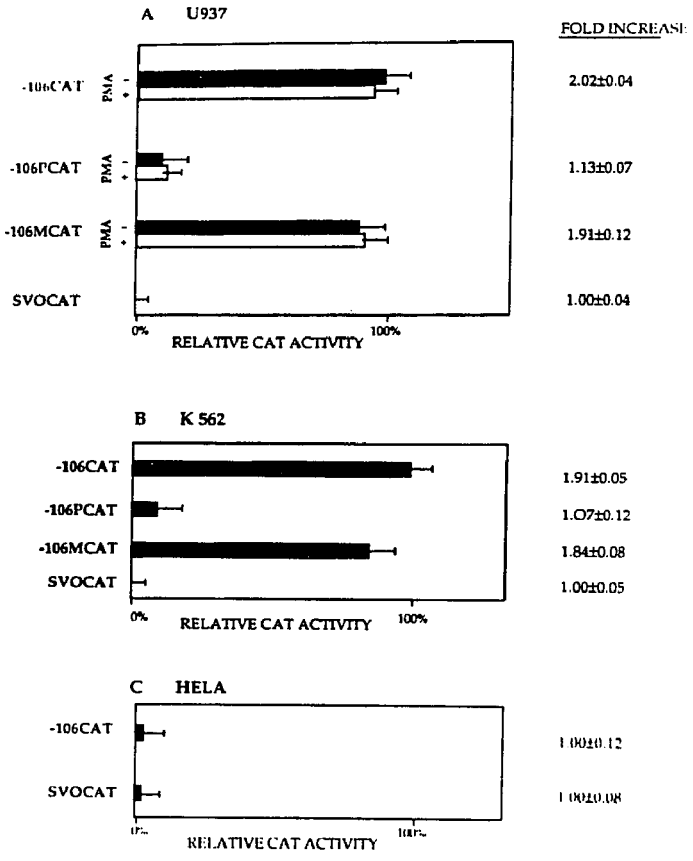

A U937
FOLD INCREASE
-106CAT
PMA
-106PCAT
PMA
-106MCAT
PMA
SVOCAT
2.02±0.04
1.13±0.07
1.91±0.12
1.00±0.04
0%
100%
RELATIVE CAT ACTIVITY
B K 562
-106CAT
-106PCAT
-106MCAT
SVOCAT
1.91±0.05
1.07±0.12
1.84±0.08
1.00±0.05
0%
100%
RELATIVE CAT ACTIVITY
C HELA
-106CAT
SVOCAT
1.00±0.12
1.00±0.08
RELATIVE CAT ACTIVITY

Fig. 20. Immunoblot of nuclear extracts from various myeloid cells. 30μg of nuclear extracts from different myeloid cells were separated, blotted and probed with a 1:1000 dilution of antiserum 9794 containing antibodies to PU.1. The primary antibodies were washed with TBS-T, bound with protein A-biotin and labeled with streptavidin-alkaline phosphatase. PU.1 related complexes were identified by color development with NBT and BCIP. Several proteins of 32-44 kDa, 80 kDa and 18 kDa were recognized by anti-PU.1 antibodies. These proteins were found in the extracts from reticulocyte synthesized PU.1 (lane 2), K 562 (lane 3), U 937 (lane 4), PLB-985 (lane 5), PLB-985 treated with cAMP (lane 8) and PLB-985 treated with PMA (lane 9). Bacterial PU.1 was identified as a single band of 38 kDa (lane 7, positive control).

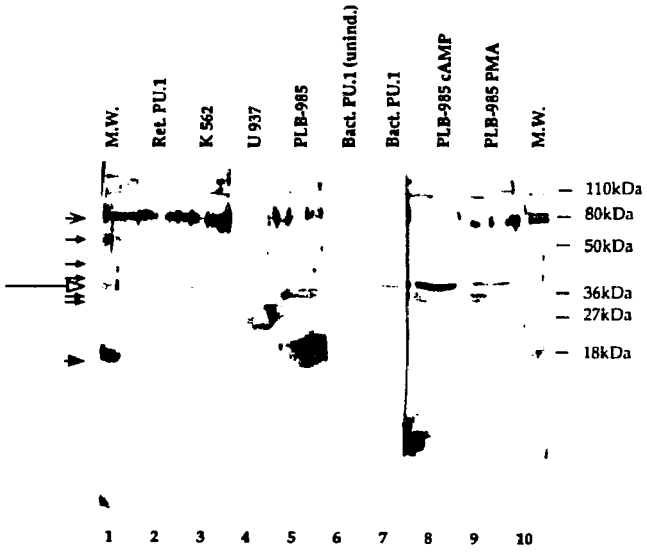

M.W.
Ret. PU.1
K 562
U 937
PLB-985
Bact. PU.1 (unind.)
Bact. PU.1
PLB-985 cAMP
PLB-985 PMA
M.W.
110kDa
80kDa
50kDa
36kDa
27kDa
18kDa
1
2
3
4
5
6
7
8
9
10

Fig. 21. South-western analysis of nuclear extracts from various myeloid cells. 50μg of nuclear extracts were separated, blotted, renatured and probed with labeled 30 bp probe. Nonspecific competitor at 10mg/ml of binding solution was added to compete away non-specific DNA binding proteins. The 30 bp probe was bound by at least four proteins of 27, 36, 38 and 46 kDa present in reticulocyte lysate containing PU.1 (lane 7) whereas no proteins were observed to bind to the probe in unprogrammed reticulocyte lysate (lane 8). These proteins were also observed in PLB-985 (lane 2), PLB-985 cells treated with cAMP (lane 3), PLB-985 cells treated with PMA (lane 4), U 937 cells (lane 5) and in U 937 cells treated with PMA (lane 6). In addition, proteins of 18-25, 55-60 and 85-95 kDa were observed in all nuclear extracts but not in reticulocyte lysates. The proteins that are PU.1 related are indicated by the bracket.

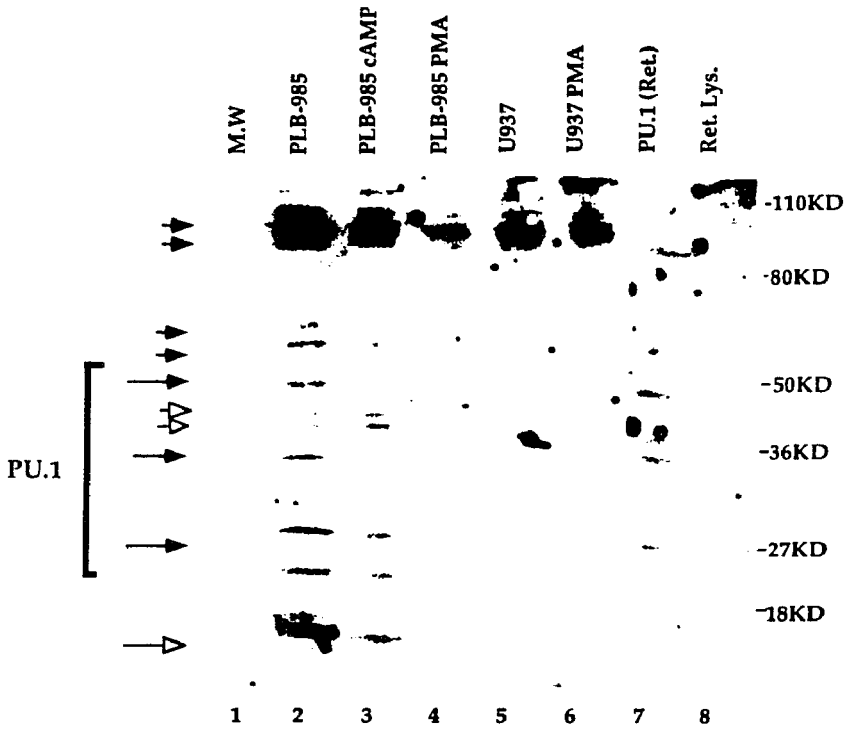

M.W
PLB-985
PLB-985 cAMP
PLB-985 PMA
U937
U937 PMA
PU.1 (Ret.)
Ret. Lys.
-110KD
-80KD
-50KD
-36KD
-27KD
-18KD
PU.1
1
2
3
4
5
6
7
8

Fig. 22. Affinity purification of the proteins that bind the 30 bp element. 100μg of nuclear extracts from PLB-985 cells was bound to agarose-immobilized 30 bp DNA in the presence of 100ng/μl of poly (dI-dC) at 4°C for 2 hours. The agarose beads were washed with six changes of binding buffer and the bound proteins eluted in the sample loading buffer. Increasing amounts of the eluate (2-10μl) were loaded on SDS-PAGE, separated and silver stained. Lane 2 containing 10μl of the eluate shows proteins of 18, 34-38, 55-60 and 85-90 kDa that are specifically bound to the sequence within the 30 bp element. The input protein is shown in lanes 7 and 8. Marked enrichment of the proteins that specifically bound to the 30 bp element was achieved using the DNA affinity column and these proteins correspond to the proteins observed to bind to the 30 bp element in south-western analyses.

PLB-985 EXTRACT

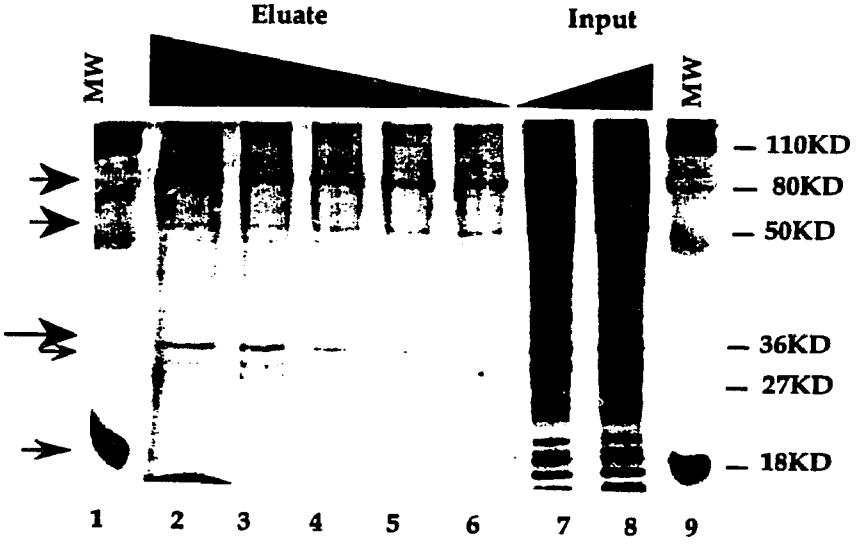


Fig. 23. DSP cross-links various complexes observed in PLB-985 nuclear extracts. Varying concentrations of DSP were added to binding reactions containing PLB-985 extract and the reactions were subject to EMSA analysis as described in Fig. 2. At 0.75 mM DSP (lanes 3 and 4), the complexes were completely cross linked and could not be resolved on a 10% nondenaturing gel. Addition of 1µl of100mM DTT resulted in the reversal of the crosslinks and all the complexes A to F were seen in the EMSA reaction (lane 2). As controls, reticulocyte synthesized human PU.1 was subjected to identical treatment (lanes 10 and 11) and no cross linked complexes were observed in these reactions. PLB-985 EMSA patterns are shown in lanes 1 and 8. Additional complexes indicated by double arrows were observed only when the reactions were performed in binding buffer containing 10mM HEPES.

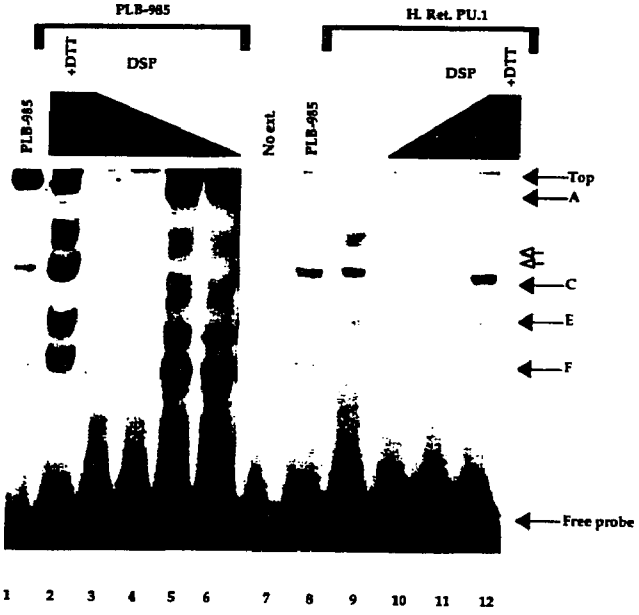
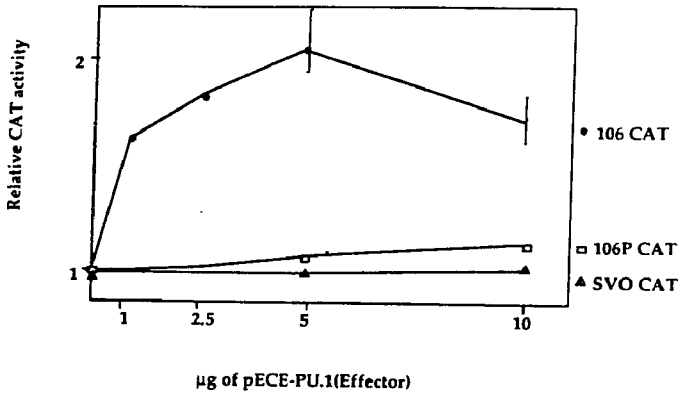
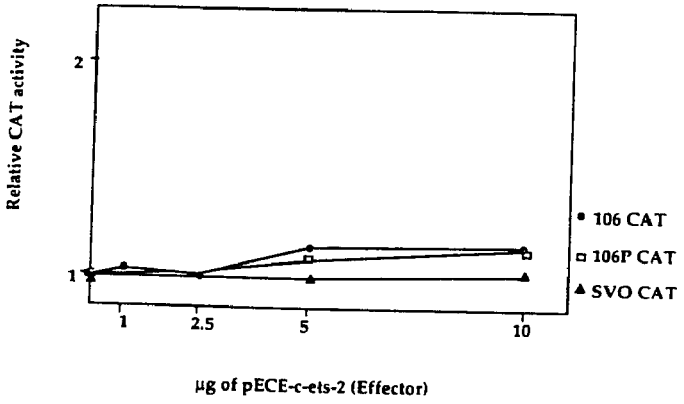

PLB-985
H. Ret. PU.1
+DTT
DSP
DSP
+DTT
PLB-985
No ext.
PLB-985
Top
A
C
E
F
Free probe
1 2 3 4 5 6 7 8 9 10 11 12

Fig. 24. Far-western analysis of nuclear extracts with labeled human PU.1. Twenty micrograms of nuclear extracts were separated, blotted, renatured and probed in TNE-50 buffer containing 2mg/ml BSA. PU.1 was labeled with ^{35}S methionine, purified and added to the binding solution and allowed to bind at 4°C for 8 hours. The blot was washed and exposed for 16 hours by autoradiography. As controls, blots were probed under identical conditions with reticulocyte lysates containing ^{35}S methionine but not PU.1. Labeled PU.1 interacted with at least 10 different proteins of different molecular weights present in nuclear extracts from PLB-985 (lane 6), K 562 (lane 7), U 937 (lane 8) and HeLa (lane 9) but did not interact with any proteins in reticulocyte lysates programmed to synthesize PU.1 (lane 5). Reticulocyte lysates without PU.1 mRNA did not bind to any proteins in nuclear extracts from PLB-985 (lane 1), U 937 (lane 2) and HeLa (lane 3).

PLB-985
U937
HeLa
M.W.
Ret.lys.PU.1
PLB-985
K562
U937
HeLa
M.W.
– 109 kD
– 80 kD
– 50 kD
– 35 kD
– 27 kD
– 18 kD

Fig. 25. Transactivation of HNE promoter by PU.1 in HeLa cells. 20μg of HNE-106 CAT, HNE-106PCAT and SVOCAT were transfected with varying amounts of effector plasmids encoding PU.1, c-Ets-2 or no gene and 0.5μg of CMV-luciferase as internal control. After 48 h., the CAT and luciferase activities were measured and the CAT values standardized to equal luciferase levels.
Panel A shows the results of transfection of pECE-PU.1 with the various HNE promoter constructs into non myeloid HeLa cells. 5μg of pECE-PU.1 activated the -106CAT construct about 2 fold similar to the levels seen in U 937 cells but failed to effect transcription from the -106PCAT construct. pECE-PU.1 did not increase activity from the promoterless SVOCAT.
Panel B. When pECE-c-Ets-2 was transfected along with the HNE promoters, no significant increase in normalized CAT values were observed for any of the promoters.


Relative CAT activity
2
1
1
2.5
5
10
• 106 CAT
□ 106P CAT
▲ SVO CAT
µg of pECE-PU.1(Effector)


Relative CAT activity
2
1
1
2.5
5
10
• 106 CAT
□ 106P CAT
▲ SVO CAT
µg of pECE-c-ets-2 (Effector)

SUMMARY

Our results from the study of regulation of the Human Neutrophil Elastase gene expression clearly show that a 30 bp element, located between -76 and -106 of the HNE promoter, is the main determinant of myeloid specific activity of the HNE promoter. The deletion of this element from the HNE promoter results in 60-85% loss of promoter activity in myeloid cells. This element binds several myeloid specific proteins that are present in all myeloid cells. These proteins of 31, 38 and 55 kDa appear to be the proteins responsible for the myeloid specific activity of the HNE promoter. Analysis of the DNAse I protection assays shows that the sequences within the 30 bp element are protected from digestion only in myeloid but not in non-myeloid cells. The TATA element and the CCAAT element, present at -153, are also protected from digestion. Functional analysis confirms that no other elements located within the first 1000 bp of the HNE promoter contributes significantly to myeloid specific activity. This 30 bp element is conserved and contains a PU.1/Ets binding motif.

Mutation of the PU.1 binding site affects the binding of various complexes to the 30 bp element. Antiserum against PU.1 interacts with the complexes observed in EMSA in the presence of myeloid nuclear extracts. *In vitro* synthesized PU.1, a B cell and macrophage specific transcription factor, binds to the Ets binding motif within the 30 bp element. Phosphatase treatment of nuclear extracts alters the EMSA pattern and indicates that one complex C is a highly phosphorylated form of PU.1. The 30 bp element binds several proteins that are different from PU.1 and there are protein-protein interactions between PU.1 and other nuclear factors. We demonstrate that differentiation of U 937 and PLB-985 cells does not alter steady state levels of PU.1 protein and, hence, no difference was observed in the activity of HNE promoter in differentiated U 937 cells.

PU.1, cotransfected into non-myeloid HeLa cells, activates the expression of the HNE promoter and clearly proves that PU.1 is the factor responsible for myeloid specific expression of the neutrophil elastase gene.

Recent studies on the regulation of normal hematopoiesis *in vitro* by PU.1 using competition of PU.1 results in the inhibition of differentiation of hematopoietic progenitors (1). This suggests a crucial role for PU.1 in the regulation of normal hematopoiesis. Additional studies using targeted disruption of the murine PU.1 gene in mice has shown that abolition of PU.1 function in early development results in embryonic lethality and marked disruption of embryonic hematopoiesis (2). There was a defect in the generation of progenitors for B and T lymphocytes, monocytes and granulocytes but not in the progenitors for erythrocytes and megakaryocytes (2). The defect in B lymphocytes and monocytes may be anticipated since PU.1 has been known to regulate the function of several B lymphocyte and monocyte specific genes (3-6). Here, we provide evidence that HNE is the first granulocyte specific gene regulated by PU.1. Since HNE is expressed at early stages of myelopoiesis, any disruption in the expression of genes expressed at this stage results in lack of differentiation of progenitors to the granulocyte lineage.

If PU.1 is involved in the regulation of genes expressed in different hematopoietic lineages, the process by which aberrant or leaky expression of lineage specific genes is mediated is undoubtedly very complex. Additional regulatory sequences and protein-protein interaction between PU.1 and other regulatory proteins may be one of the mechanisms by which this complex regulation is mediated. Our future efforts are aimed at characterizing additional sequences required for the lineage and stage restricted expression of the HNE gene. Our efforts are directed at determining the sequences in the 5' and 3' flanking regions that confer high level expression to the HNE promoter. HNE exists in a locus of three promyelocyte specific serine protease genes. These include the genes for Azurocidin, Proteinase 3 and Neutrophil Elastase (7). Considerable similarity

exists in the promoters of these three genes and the possibility exists that stage and tissue specific hypersensitivity sites govern the overall regulation of the genes at this locus. We are interested in determining if such global regulatory mechanisms exists at this locus and wish to examine at least 50 kb of 5' and 3' flanking sequences at this loci. Such locus control region and hypersensitivity sites have been reported to exist in other clusters of genes like the β-globin locus and have been shown to play a crucial role in the high level coordinated expression of genes at this locus (8,9).

The molecular events that govern the orchestrated events occurring during myelopoiesis are slowly being unraveled. For example, down-regulation of the mRNA for myeloblastin results in the induction of differentiation of myeloid cells although the exact mechanisms by which this occurs is still not clear (10). It is apparent that the cellular machinery and regulatory mechanisms governing the phenotypic differentiation state of myeloid cells monitor the molecular events involving the stage specific expression of myeloid restricted genes. Our studies described here dissect one particular molecular event in the process of differentiation of myeloid cells and sheds insight into the processes involved into the coordinated events of myelopoiesis. Our future studies will further our understanding of the molecular events in normal myelopoiesis and help gain insights into the processes that result in aberrant expression and leukemogenesis of myeloid cells.

LIST OF REFERENCES

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