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# The myeloid-lineage specific enhancer of the mouse myeloperoxidase gene consists of three *cis*-elements defined as in vitro DNase I footprints

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**Abstract**

The myeloid-lineage specific enhancer at 3.4–3.1 kb upstream of the mouse myeloperoxidase gene [1] has been further characterised. In vitro DNase I footprinting experiments revealed three protected sequences (FT-I, -II and -III) in the enhancer, associated with the proteins that are enriched in WEHI 3BD$^+$ cells, at which the MPO gene is highly expressed; but not in two non-MPO expressing lymphocytic cell lines. Site-specific mutations at each element severely reduced the level of the reporter gene activity in a non-additive manner. This is parallel with either abolishment or alteration of the corresponding wild-type protein–DNA interaction in vitro. Consideration of the sequence motifs present in the enhancer suggests that the *cis*-elements defined as the in vitro DNase I footprints are likely to be novel.

*Key words:* Myeloperoxidase; Myeloid-lineage specific enhancer; DNase I footprint (in vitro); *cis*-element

## 1. Introduction

Haemopoietic stem cells have the developmental potential for at least 8 or 9 lymphoid and myeloid lineages. The process of the myeloid cell differentiation, which converts multipotential progenitor cells into one of six mature differentiated cells, i.e. erythrocytes, platelets (megakaryocytes), macrophages, neutrophils, eosinophils and basophils, involves temporally regulated expression of a number of lineage- and stage-specific genes. Understanding the developmental specification of lineage and maturation stage associated patterns of gene expression in haemopoiesis requires insight into the control of individual lineage-specific 'marker' genes for each lineage. Within the myeloid lineages, extensive studies have described a complex interplay of regulatory *cis*-elements and binding nuclear proteins, in particular for the $\beta$-globin enhancer 5′ locus control region in the erythrocytic lineage [2]. In this respect, there is, with a few recent exceptions [3–5], a marked deficit of information on the granulocytic lineage, a major pathway of non-erythroid myeloid cell development.

The myeloperoxidase gene (MPO) provides an attractive model for such studies. MPO gene expression is regulated in both a cell-lineage and differentiation-stage specific manner during granulopoiesis. The MPO protein and bactericidal function are maximally expressed at the promyelocytic stage of granulocytic differentiation; subsequently as these cells mature into granulocytes, levels of MPO mRNA rapidly decline, probably as a result of both cessation of transcription and decrease in stability of MPO mRNA [6]. In the previous report, we have identified a myeloid-lineage-specific enhancer at approximately 3.1 kb upstream of the mouse MPO gene [1] by a transient transfection assay. It functions and resides at a DNase I hypersensitive chromatin region in WEHI 3BD+ cells, a myelomonocytic cell line [7,8] at which the mouse MPO gene is highly expressed, but not in two non-MPO expressing lymphocytic cell lines (pre-B lymphocytic cell line, 18.8 [9] and pre-T lymphocytic cell line, EL-4 [10]). Functional analysis of the deletion mutants by a transient-transfection assay, suggests that the integrity of the 301 bp *BclI*/*Kpn*I fragment is required for both the function and the orientation-independence of the enhancer [1]. In order to delineate the functionally important *cis*-elements, I have attempted to identify the in vitro DNase I footprints specifically associated with WEHI 3BD$^+$ cells, and functionally assess the effect of the site-specific mutations on the enhancer function as well as the wild-type DNA–protein interaction in vitro.

## 2. Materials and methods

### 2.1. Cell cultures, and the transient transfection assay

WEHI 3BD$^+$, a myelomonocytic leukaemic cell line [7,8], 18.8 [9] (a pre-B cell line derived from BALB/c bone marrow infected with Abelson leukaemia virus, and EL-4 (a C57BL murine T lymphoma (ATCC TIB39)) cell line [10] were cultured as previously described [11]. Transient transfection by electroporation and luciferase assay were carried out as previously described [1].

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*2.2. Site-specific mutagenesis*

Site-specific mutagenesis was carried out essentially according to Deng's method [12] and using Clone-tech Transformer Site-directed mutagenesis kit. Two different oligonucleotides containing partially degenerate nucleotide sequences were used to introduce two sets of site-specific mutations within each individual footprint in En-1(Sc/K) which contains the 477 bp *ScaI/KpnI* fragment upstream of the Tk promoter in Pt-109 (Fig. 2). The identity of the mutants were confirmed by sequencing.

*2.3. Preparation of whole cell extracts and in vitro DNase I footprinting of sequence specific protein–DNA interactions*

Whole cell extracts were prepared from 2 litres of cells at a concentration of 0.4–0.8 × $10^6$/ml, essentially according to Manley's method [13]. Proteinase inhibitors were included in all solutions for the preparation and heparin chromatography of whole cell extracts, as well as in vitro DNase I foot-printing assays (2 μg/ml aprotinin, 1 μg/ml leupeptin, 1.4 μg/ml pepstatin, 0.5 mM benzamidine and 0.5 mM PMSF). The protein concentration of extracts was measured by comparing the amido black staining density of dotted, dried protein fractions with that of a series of dilution of bovine serum albumin on nitro-cellulose paper [14]. Both heparin chromatography of the whole extracts and in vitro DNase I footprinting assay were performed essentially as described by Dynan and Tjian [15].

## 3. Results

### *3.1. Three WEHI 3 BD⁺ cell type-specific in vitro DNase I footprints within the MPO gene enhancer*

The in vitro DNase I footprinting assay [15] for the sequence-specific DNA–protein interactions was used to identify the candidate *cis*-elements within the upstream enhancer of the mouse MPO gene. The *BamHI/XhoI* fragment from En-1(S/K) was 3′ end-labelled at the *XhoI* site as the probe. As shown in Fig. 1A, there are three footprints (FT-I, -II and -III) associated with the proteins in 10 μl (37 μg protein) whole cell extract from WEHI 3BD⁺ cells, but not with 25 μl (138 μg proteins) whole cell extracts of EL-4 cells and 25 μl 0.4 M KCl elute (15 μg protein) of the heparin chromatography of the whole cell extract of 18.8 (N.B. a nuclease activity was observed in the whole cell extract of 18.8, and can be eliminated in the 0.4 M KCl elute from the Heparin affinity chromatography of the whole cell extract). Being aware of the possibility that the absence of these footprints may be caused by the unsatisfactory quality of the protein extracts from both 18.8 and EL-4 cells, I have carried out an in vitro DNase I footprinting analysis of a 1082 bp *SalI-EcoRI* fragment which contains both promoter and enhancer of the SV 40 early gene (418 bp *HindIII/HpaII* fragment) from plasmid pSG5 [16] and 10 μl extracts from each cell line, respectively. The region, between 185 and 260 nucleotides from the *SalI* site consists of six GC boxes, which are recognised by a ubiquitously expressed transcription factor, Sp-1 [17] and is protected to a comparable extent from DNase I digestion by the proteins from all the three cell lines (Fig. 1C). Thus, both the quality and the Sp-1 activity of the protein extracts from WEHI 3BD⁺, 18.8 and EL-4 cells are compatible. Therefore, the proteins responsible for the occurrence of footprints (FT-I, -II, and -III) in this enhancer are significantly more abundant in WEHI 3BD⁺ cells. These proteins are either absent or too rare to be detected by the assay in both El-4 and 18.8 cells.

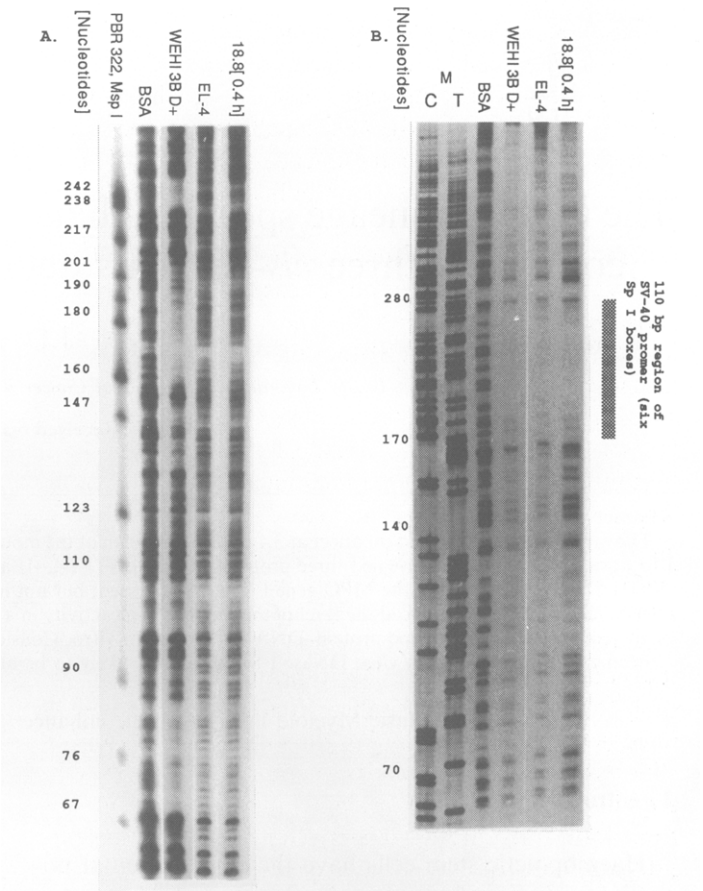


Fig. 1. WEHI 3BD⁺ cell-type-specific in vitro DNase I footprints (A). The 490 bp *BamHI-XhoI* fragment, containing the 477 bp *ScaI-KpnI* MPO enhancer fragment, was 3′ end-labelled at the *XhoI* site (the sense strand is analysed). The DNase I footprinting experiment was carried out with a varying amount of whole cell extract from WEHI 3BD⁺ cells (10 μl, 37 μg protein), and EL-4 cells (25 μl 137 μg protein) and a 0.4 M KCl elute from heparin chromatography of the whole cell extract of 18.8 cells (25 μl, 15 μg protein). The control was with 12. 5 μg bovine serum albumin. Three DNase I footprints are indicated by the shaded box on the left side of the autoradiograph. The size marker was provided by end-labelled *MspI* digests of PBR 322 plasmid DNA. (B) A 1024 bp *SalI/EcoRI* fragment of pSG5 [16], (six Sp I motifs are located from 185 to 260 bp from the *SalI* labelled site) was 3′ end-labelled (antisense strand is labelled) and used as the probe for in vitro footprinting. 10 μl of extract of WEHI 3BD⁺, EL-4 and 18.8 cells were used. This fragment contains the 418 bp *HindIII/HpaII* segment of SV 40 enhancer and early promoter (0.725–0.648 m.u.) of SV 40 DNA and the rabbit β-globin intron II. The footprint (approximately 110 bp long containing six Sp I sites is marked on the right of the autoradiograph. The size marker is provided by a sequencing ladder from a known sequence.

### *3.2. Site-specific mutations at the sequences of each footprint impair the enhancer function*

To search for the function of the *cis*-elements defined as the WEHI 3BD⁺ cell-type-specific footprints (Fig. 1)

in the enhancer, I mutated the sequence of each footprint and assessed the mutations' effects on the enhancer function by a transient transfection assay. As each footprint (16 to 40 bp in length, Fig. 4) is capable of accommodating two or more individual sequence-specific DNA–protein interactions, I have as a preliminary characterisation of the enhancer, used two oligonucleotides, consisting of different sets of degenerate sequences to individually introduce two sets of mutations at each footprint into the construct, En-1(Sc/K) (Fig. 2). The luciferase expression in the transfected WEHI 3BD$^+$ cells by each mutant was measured in parallel with that of Pt-109 and En-1(Sc/K). The enhancement in the luciferase expression by all mutants tested was reduced drastically in comparison to that by En-1(Sc/K). Among the mutants tested, two different types of mutation at each footprint for further analysis are listed in Fig. 2. The enhancement in the luciferase expression by the mutants ranged from 1.5- to 15-fold, in comparison to 105-fold by the En-1(Sc/K), over the luciferase expression by Pt-109. It is clear that the contribution of the DNA–protein interactions at each footprint to the enhancer function cannot be defined in quantity, according to the simple arithmetic role. This indicates that a synergistic action contributed by the sequence-specific DNA–protein interactions at all the three footprints through a complex and yet to be defined interplay is required for the enhancer function.

### 3.3. *The wild-type DNA–protein interactions were affected or altered by these mutations*

The further evidence for a direct contribution of the *cis*-elements defined as footprints to the enhancer function would come from the experiments to assess the mutations' effects on the wild-type DNA–protein interactions in vitro. Bearing in mind the possibility that the mutations' effect on the enhancer function (Fig. 2) might be contributed by a new type DNA–protein interaction being fortuitously created by the mutations, I used the in vitro DNase I footprinting assay to examine the DNA-protein interaction patterns of the whole cell extract of WEHI 3BD$^+$ cells with the enhancer fragments from the mutated En-1(Sc/K) ($\Delta$1 and $\Delta$1a4 for FT-I; $\Delta$11 and $\Delta$2a12 for FT-II; and $\Delta$3b15 and $\Delta$3c6 for FT-III).

Although the mutations were created at two different parts of FT-I in $\Delta$1 and $\Delta$1a4, respectively, their effect on both the function of enhancer (6.5- and 5-fold enhancement, respectively; Fig. 2) and the DNA–protein interactions (FT-I was abolished; Fig. 3B) are very similar. At mutants $\Delta$11 and $\Delta$2a12 for FT-II, the sequence at the centre and 3′ part of FT-II was changed, respectively. As shown in Fig. 3E, the mutated part and upstream part of FT-II in $\Delta$11 were not protected from DNase I cleavage, while the rest of the footprint remained intact. In $\Delta$2a11, the mutated 3′ part was not protected, while the remaining part was fully protected from the DNase I digestion. Thus, in FT-II, there are at least two separable motifs recognised by protein(s) in WEHI 3BD$^+$. However, although mutations at $\Delta$11 and $\Delta$2a12 had very different effects on the DNA–protein interaction at FT-II, their effect on the function of the wild-type enhancer was comparable (10- and 15-fold enhancement, respectively; Fig. 2). In the case of FT-III, in $\Delta$3b15, the central 10 bp was mutated, while in $\Delta$3c6, the whole FT-III was affected. Both mutants brought the enhancement from 105-fold of the wild-type enhancer down to 1.5- and 1.9-fold levels, respectively (Fig. 2) and abolished FT-III (Fig. 3D). It was also observed that the protein–DNA interactions at the wild-type footprints in each mutant (for instance, both FT-II and -III in mutants $\Delta$1 and $\Delta$1a4) were not affected.

## 4. Discussion

### 4.1. *A synergistic action of FT-I, -II and -III is required for the function of the MPO gene enhancer*

The transcriptional regulation of eukaryotic genes involves the combinatorial effects of both lineage-restricted and common or ubiquitous transcription factors [18]. The effect of more than one *cis*-element (their cognate transcription factors) on the transcription of a gene is often synergistic rather than additive [19]. There is

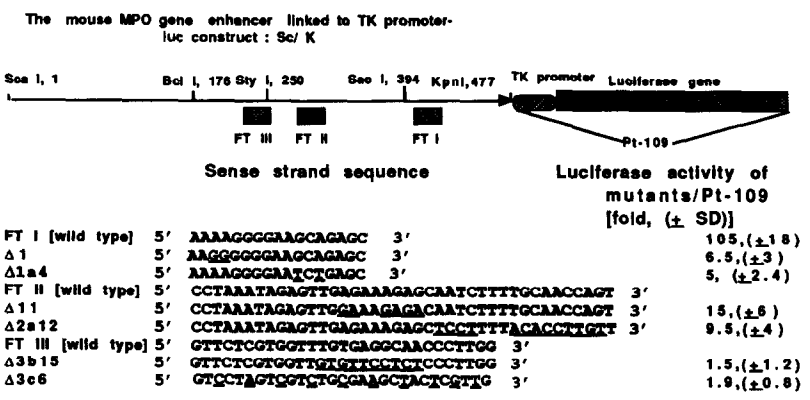


Fig. 2. Site-specific mutations at the sequence in each footprint and their effects on the luciferase expression in the transfected WEHI 3BD$^+$ cells.

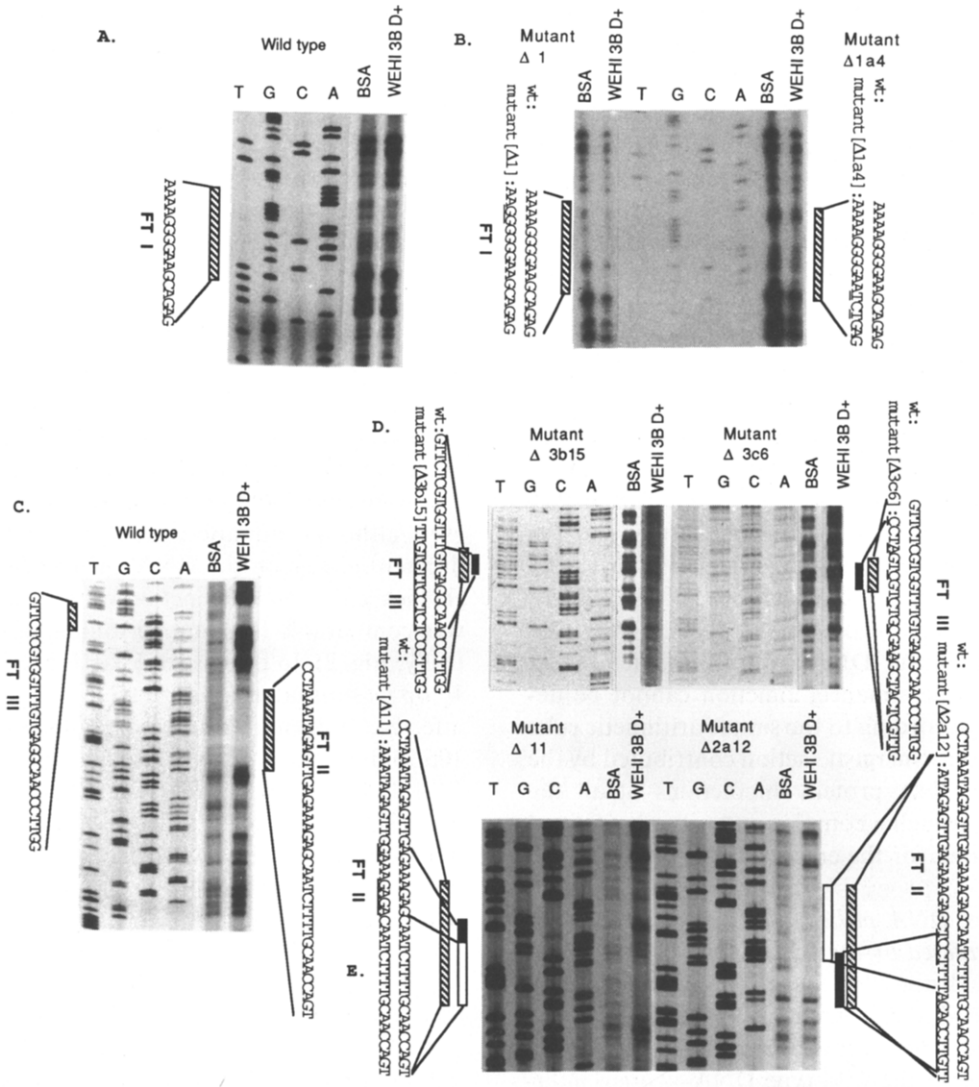


Fig. 3. Site-specific mutations and their effects on the corresponding in vitro DNase I footprints 10 μl of the whole cell extract of WEHI 3BD$^+$ cells was incubated with the single end-labelled *Bam*HI-*Xho*I fragment of En-1(Sc/K) and *Apa*I/*Xho*I fragments (containing the 471 *Sca*I-*Kpn*I fragment) of the following mutants: Δ1 and Δ1a4 (FT-I), Δ11 and Δ2a12 (FT-II) and Δ3b15 and Δ3c6 (FT-III) for the in vitro DNase I footprinting analysis. A sequence ladder was used as a reference for the assignment of the sequence of each footprint, for which the primer had its 5′ end complimentary to the $^{32}$P-labelled end of the probe described in (A). The sense strand sequence of the footprints are indicated. N.B. Only the results concerning the affected footprints is shown, as the rest footprints remain intact in all the cases tested. (A) The in vitro DNase I footprint pattern of the FT-I in the wild-type template. (B) The absence of FT-I in the mutated templates, Δ1 and Δ1a4. N.B. Only the sequence ladder of Δ1 is shown. (C) The in vitro DNase I footprint pattern of the FT-II and FT-III of the wild-type template. (D) The absence of FT-III in the mutated templates, Δ3b15 and Δ3b6. (E) The absence of FT-II in the mutated templates, Δ11 and Δ2a12. The original footprint regions over the wild-type sequence are shown by shaded bars, and the footprints over the mutants by open bars The filled bars show the mutated regions.

such an example in myeloid lineage. It has been shown the lineage-specific expression of the chicken *mim-1* gene seems to be determined by a synergistic action of myb (widely expressed in haemopoietic cells) and NF-M (a myeloid-specific member of the C/EBP family of transcription factors) [4,5].

The first indication for the synergistic action of the *cis*-elements of the three footprints (FT-I, -II and -III) responsible for the function of the MPO gene enhancer was from a function analysis of a set of deletion mutants [1]. The impairment of the integrity of the 301 bp *Bcl*I/*Kpn*I fragment by deletion from either end have severely reduced the enhancement in a non-additive manner [1]. In this report, I have shown that the mutations affecting the DNA–protein interactions at any one of the three footprints impaired the enhancer function in a similar non-additive manner (Fig. 2). Furthermore, all the deletion mutants which have deleterious effects on the enhancer function, described in the previous report [1], lack, at least, one of the three footprints (Figs. 1 and 2). Therefore, the *cis*-elements defined as the WEHI 3BD$^+$ cell-type-specific footprints are the integral components

of the enhancer, and a synergistic action involving all the footprints is essential for the enhancer function. Since only the DNA–protein interactions at the mutated footprints were affected (Fig. 3, and data not shown), the protein–protein interactions between the specifically bound proteins of the three footprints which is likely to be also affected by the mutations at any one of the three footprints, may be the key event of the enhancer activity.

### 4.2. *The novelty of the* cis*-elements in the MPO gene enhancer?*

Since a large number of the *cis*-elements have been identified and their cognate transcription factors have been recently characterised and cloned [20–22], the novelty of the newly discovered *cis*-elements can be quickly assessed by the sequence homology analysis with a computer assistance. Three consensuses respectively bound by the previously characterised transcription factors, e.g. Sp-1, myb and PEA3, are found in the MPO gene enhancer (301 bp *BclI/KpnI* fragment; Fig. 4). However, irrespective of the MPO gene expression, the binding activity of Sp-1 is compatible among the cell lines studied (Fig. 1C, and Fig. 3A). The messenger level of Myb is also indistinguishable among the three cell lines by Northern analysis (unpublished data). PEA3 is not expressed in haemopoietic cells [23]. Therefore, these three consensuses and their cognate transcription factor are not likely to make, if any, direct significant contribution to the enhancer function. Furthermore, these consensuses are not protected by the proteins of all the three cell lines from DNase I digestion in vitro (Fig. 1). Therefore, the identification of the motifs based on a sequence homology does not automatically demonstrate their functional importance, as recently critically discussed by Mikkelsen [24]. However, the lack of a sequence homology with the *cis*-elements bound by the previously described transcription factors [21–23], does suggest that the *cis*-elements defined as FT-I, -II, and -III (Fig. 4), recognised by the proteins highly enriched in WEHI 3BD$^+$ cells might be novel. Current effort to define the direct nucleotides contact at each footprint (FT-I, -II and -III) by using the approaches such as methylation interference [25] and in situ OP-Cu footprinting [26], will not only provide an insight into the nature of the *cis*-elements (FT-I, -II and -III) and their cognate transcription factors, but also pave the way for cloning the transcription factors involved.

### 4.3. *The upstream enhancer of the mouse MPO gene and the promyelocytic stage-specific expression of the mouse MPO gene*

The WEHI 3BD$^+$ cell type specificity of the upstream enhancer of the MPO gene has been demonstrated by the comparative studies of its DNase I hypersensitivity in nuclei [1], the expression of the MPO mRNA [11], the function by a transient transfection assay (Figs. 2 and 3), as well as the occurrence of the in vitro DNase I footprints (Fig. 1). Since, all the comparable analyses with the two non-myelocytic controls (18.8, for pre-B, and EL-4 for pre-T lymphocytic) are negative [1], and the lineage identity of all three cell lines are well documented [7–10], this enhancer is likely to be specific to the neutrophilic granulocytic lineage on the myeloid differentiation pathway.

The fact that the MPO gene is expressed at the premyelocytic stage of granulopoiesis in vivo, raises an interesting possibility: the MPO gene enhancer may also be specific for the promyelocytic stage. Indeed, there are some supportive preliminary data. The extracts from the several non-MPO expressing myeloid cell lines, including MEL cells (an erythrocytic cell line [27]), WEHI 3BD$^-$ cells (a myelomonocytic cell line [7]), and 416 B cells (a myeloid progenitor cell line [28]), gave rise to none of the three footprints. On the contrary, the whole cell extract from a myelomonocytic cell line, NFS-60 [29] where the MPO gene does express, gives rise to all the three footprints (unpublished data). Since WEHI 3BD$^+$ cells have a potential to differentiate into neutrophilic granulocytes induced by granulocyte colony stimulating factor [8], accompanied with the cessation of the expression of the MPO gene, this important issue can be further addressed by testing the function of the MPO gene enhancer in the induced WEHI 3BD$^+$ cells.

Recently, another group has reported a positive *cis*-element at several hundred bp upstream of the mouse MPO gene which is only functional in a myeloid cell line, 32D Cl3, but not in fibroblastic cells [30]. Since no haemopoietic cell control has been considered when both function of the *cis*-element identified and the in vitro sequence-specific DNA–protein interactions were analy-

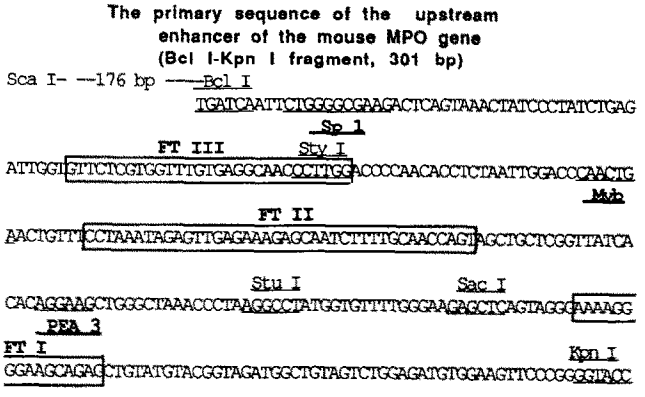


Fig. 4. The primary sequence of the upstream enhancer of the mouse MPO gene. The primary sequence of the upstream enhancer (*BclI-KpnI*, 301 bp fragment) and three in vitro DNase I footprints (FT-I, -II and -III; boxed) are shown. The sites of several relevant restriction enzymes are also indicated. Also underlined are consensuses recognised by the previously characterised transcription factors compiled in references [21–23] (the motifs were manually imported into the motif data bank in GeneWork, version 2.1 (Intelligenetics Inc., USA). The identified motifs within the 301 *BclI/KpnI* fragment were viewed in the motif view. Sp-1 (G/T)(G/A)GGC(G/T)(G/A)2(G/T) myb (T/C)AAC(G/T)G PEA3 AGGAA(A/G).

sed, the functional role of this element in the myelomonocytic lineage-specific expression of the moue MPO gene remains to be seen. However, this *cis*-element is clearly distinct from the enhancer being characterised in some detail in this report.

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