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The mouse M-lysozyme gene domain: identification of myeloid and differentiation specific DNaseI hypersensitive sites and of a 3′-cis acting regulatory element

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ABSTRACT

The mouse M-lysozyme gene is exclusively expressed in myeloid cells of the blood system being progressively turned on upon cell differentiation. In this study the mechanism controlling this tissue- and differentiation stage-specific gene expression was analyzed at the level of chromatin structure. A complex pattern consisting of constitutive and differentiation dependent DNaseI hypersensitive sites (HSs) was found in a set of various myeloid cell lines, representing different stages of maturity. The chromatin of a lymphoid cell line, which does not express the lysozyme gene, is completely insensitive to DNaseI digestion. Chromatin analysis of two multipotent hematopoietic stem cell lines which can be differentiated *in vitro* to mature myeloid cells confirmed that these identified DNaseI HSs are specific for distinct differentiation stages, rather than being a characteristic feature of the cell lines. Additionally, the stem cell studies revealed that the hypersensitivity of the chromatin domain is already established at the multipotent stage. DNA fragments spanning a cell type- and differentiation stage-specific cluster of HSs in the 3′ region of the gene showed enhancer activity in all cell types tested. In the light of this lack of specificity, we suggest that cell type-specific modification of the chromatin structure in this region may play a role in determining the binding of a widespread transcription factor, and hence contribute to the time specificity of lysozyme M gene expression.

INTRODUCTION

The mouse lysozyme P- and M-genes are the products of a gene duplication event approximately 50 million years ago. These closely related lysozyme genes are expressed with different tissue specificities (1). The P-gene is active in paneth cells of the small intestine where the gene product is presumably used for digestive purposes. Expression of the M-lysozyme gene, on the other hand, is restricted to haemopoietic cells, specifically macrophages and granulocytes. Here, the expression correlates with maturation in that the gene is progressively turned on during differentiation from the myeloid precursor cell to the mature macrophage, where it is fully active (2). Our present studies have concentrated on the molecular mechanisms involved in tissue specific and development stage-specific expression of the mouse M-lysozyme gene.

Preferential cleavage by nucleases at specific sites of the chromatin has been a useful tool for monitoring gene activity at the level of chromatin organization. Active genes are packed in an altered chromatin structure, forming a domain of overall nuclease sensitivity (3). This structure contains specific sites that are hypersensitive to DNaseI digestion, and which may correspond to sites of DNA modification, or binding of specific non-histone proteins. These DNaseI hypersensitive sites (HSs) often correlate with binding sites for regulatory proteins (see for reviews: 4–7). Tissue specific changes in chromatin structure of a variety of genes have been studied in the past mainly by comparing chromatin structures of different tissues or different cell lines. To study the control of the mouse M-lysozyme gene expression, we determined the organization of HS pattern in the M-lysozyme gene domain in various myeloid cell lines which represent different stages of myeloid differentiation, and also in a non-expressing T-cell line. In addition, we have examined changes in the pattern of HS sites in multipotential haemopoietic stem cell lines that can be induced to undergo differentiation and development to produce mature macrophages and neutrophils (8, 9). These cell lines, called FDCPmix, have a dipoloid karyotype, are non-leukaemic, and their continued growth (and differentiation) *in vitro* requires the presence of physiological regulators of haemopoiesis: the colony stimulating factors. In the

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presence of interleukin-3 (IL-3) the cells undergo self-renewal as multipotential stem cells and show litle or no spontaneous differentiation to mature cells. When the IL-3 concentration is reduced and a combination of granulocyte/macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) is added to the culture medium, proliferation in association with loss of multipotentiality, and differentiation of the cells to progenitor cells of the neutrophil/macrophage lineage are observed. Further proliferation of these cells then results in the production of post-mitotic, phenotypically mature macrophages and neutrophils with associated loss of clonogenic potential. As such, the FDCP-mix cells provide a powerful system in which to examine gene control during haemopoietic stem cell differentiation and development.

Here we describe a complex, differentiation stage-specific pattern of HSs around the lysozyme M gene. In addition, functional analysis of a prominent, differentiation specific clustered array of HSs in the 3'-region revealed a strong positive regulatory effect in all cell lines tested.

This study shows firstly that changes in the DNase I hypersensitivity of chromatin in differentiating cell are remarkably complex, and secondly that alterations in chromatin structure may serve to regulate the tissue-specific function of ubiquitous transcription factors.

MATERIALS AND METHODS

Tissue culture

The mouse myeloblast cell line RMB1, immature macrophage cell line Wehi 3b, mature macrophage cell line P388D1 (10), and the fibroblast cell line NIH 3T3 were grown in Dulbecco's modified Eagle's medium supplemented with fetal calf serum to 10%. For the mouse T-Lymphoma cell line El4, RPMI medium supplemented with 10% fetal calf serum was used. The mature macrophage cell line J774.16 was grown in Duldecco's modified Eagle's medium supplemented with 20% horse serum, 5% NCTC, 1% non essential aminoacids and 1% glutamine. The mouse hematopoetic stem cell lines FDCP-mix A4 and FDCP-mix C2GM (hereafter denoted FDCP-A4 and C2GM) were maintained in Fisher's medium containing 20% horse serum and medium conditioned by the growth of Wehi 3B cells as a source of IL-3. In these conditions, the cells undergo self-renewal and little or no spontaneous differentiation (9). For inducing myeloid cell differentiation these cells were grown for 7 to 8 days in Iscove's medium suppplemented with 15% fetal calf serum and 1.5 u recombinant (r)Il3, 1000 u r.G-CSF, 50 u r.GM-CSF (each per ml) and 15% L cell conditioned medium as a source of M-CSF. Recombinant murine IL-3, human G-CSF and murine GM-CSF were gifts from Biogen, Basel, Switzerland (r.IL-3), and Amgen, Thousand Oaks, California, USA (r.GM-CSF and r.G-CSF). M-CSF was prepared using established procedures (21).

Lysozyme protein measurement

Lysozyme activity was determined using a freeze-dried suspension of *Micrococcus lysodeikticus* as substrate (0.25 mg/ml in 50 mM Na phosphate buffer pH7.0). Cell extract of 1×10^6 cells was prepared by 3 cycles of freezing and thawing, the cell debris was removed by centrifugation. After adding 50 μl of cell extract to 2 ml substrate solution, lysozyme activity was assayed photometrically at 25°C by the decrease in turbidity at 540 nm and compared as a control to the activity of purified egg-white lysozyme.

DNaseI hypersensitive site analysis

Cells in exponential growth phase were harvested at 800×g, washed once in phosphate-buffered saline, and suspended at 1×10^7 cells/ml in ice-cold homogenisation buffer (10mM Tris[pH 7.5], 15mM NaCl, 60 mM KCl, 0.15 mM Spermine 0.5mM Spermidine, 1 mM EDTA, 0.1 mM EGTA, 0.2% NP

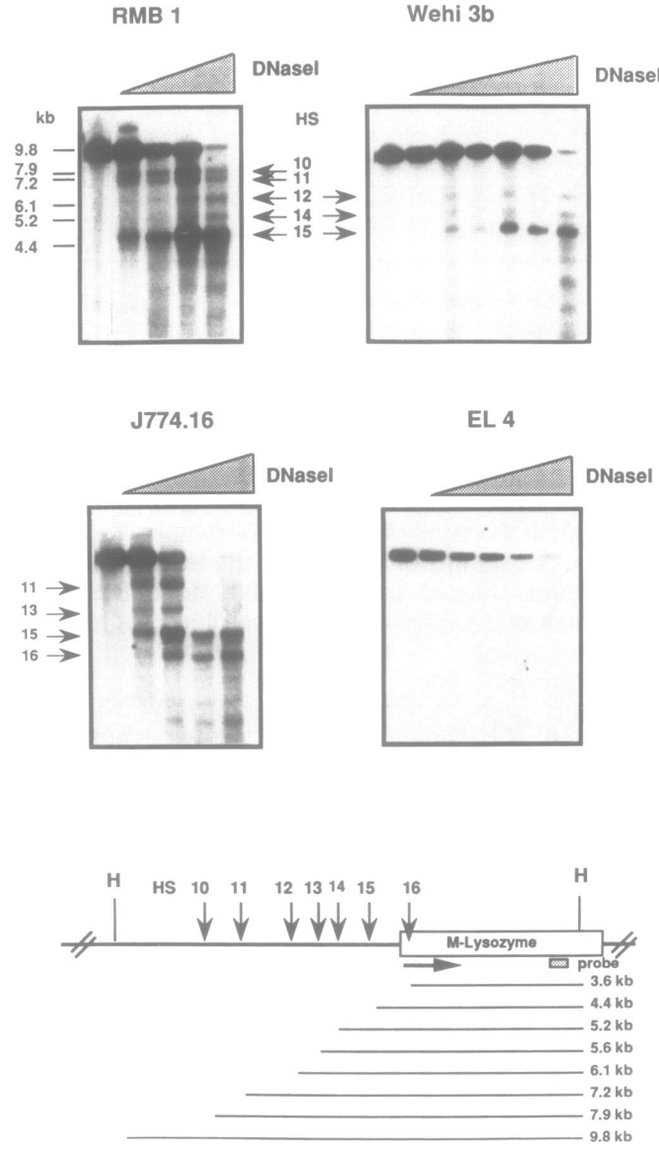


Fig.1. Mapping of DNaseI hypersensitive sites in the 5' and intragenic region of the M-lysozyme gene domain. Nuclei isolated from a myeloid precursor cell line (RMB1), immature macrophage (Wehi 3b), mature macrophage (J774.16) and a T-cell line (El 4) were incubated with increasing amounts of DNaseI (marked with a triangle). After DNA purification, 20 μg of each sample was digested with HindIII, electrophoresed on a 1% agarose gel, blotted and indirectly endlabeled with the probe shown in the diagramm below. HSs are indicated by arrows and are numbered from 10 to 16. The samples in the first lanes were not DNaseI digested. The diagram illustrates the positions of HSs. The open box represents the M-lysozyme gene (including 4 exons and the introns). The horizontal arrow under the gene marks the direction of transcription. The shaded square indicates the extension of the probe. Horizontal lines beneath the map represent the size of the parental 9.8 kb HindIII segment and the subbands (3.6 to 7.9 kb) generated by nuclease hypersensitivity. Vertical arrows on the map show the positions of DNaseI HS 10 to 16.

40 and 5% Sucrose). Cell suspensions were homogenized in a glass Dounce homogenizer with a B-type pestle, and cell lysis was monitored by phase microscopy. Nuclei were pelleted by centrifugation at 1000×g for 5 min through a sucrose cushion (homogenization buffer 10% instead of 5% sucrose) and washed once in buffer W (10 mM Tris [pH7.5], 15 mM NaCl, 60 mM KCl, 0.15 mM Spermine, 0.5 mM Spermidine). Nuclease digestion was carried out in a volume of 1 ml buffer W. Nuclei were incubated at increasing unit concentrations (generally 0–600 units/ml) of DNaseI (DPFF Worthington) for 10 min on ice. Reactions were terminated by addition of sodium dodecyl sulfate to 0.5% and EDTA to 50 mM, followed by treatment with proteinase K (100 μg/ml) at 60°C for 4 h. DNA was extracted and purified with equal volumes of phenol-chloroform isoamylalcohol (25:24:1) and ethanol precipitation. DNA (15 μg) from each sample was digested to completion with the indicated restriction enzymes and fractionated by electrophoreses in 1% agarose gels. Fragmented DNA was transferred to Hybond N filters by the method of Southern. The filters were air dried, UV-crosslinked and hybidized at 65°C overnight in hybridization buffer (0.5 M Na_2HPO_4, 7% SDS, 1% BSA, 1mM EDTA). All probes were ^{32}P labeled by random priming according to Feinberg and Vogelstein (22). Blots were washed (40 mM Na_2HPO_4, 1mM EDTA, 1% SDS) and exposed to X-ray film for 1 to 4 days. The size of the hybridizing bands appearing in the autoradiogram were determined by using a 1 kb DNA-ladder as size standard in each gel.

RNA analysis

RNA prepared from cell lines by the guanidinium isothiocyanate method was electrophoresed through 1% agarose 2.2M formaldehyde denaturing gels (23). RNA-blotting and hybridization was done as described for DNA-blotting.

Plasmid construction and cell transfection

The fragments corresponding to the region of HS 1 to 6 were generated by restriction digestion as follows: SpeI/NdeI (pHS 1 tkCAT), PvuII/KpnI (pHS 2 tkCAT), PvuII/BglII (pHS 3 tkCAT), KpnI/HindIII (pHS 4 tkCAT), PvuII/HindIII (pHS 2/4 tkCAT), HindIII/PvuII (pHS 5/6 tkCAT). These fragments were filled in with Klenow Polymerase and cloned into the filled in Bam HI side of the plasmid pBL-CAT2 (NdeI/HindIII Fragment is deleted). DNA transfections of the NIH 3T3 cells were performed as decribed by Steiner and Kaltschmidt (24). The RMB1 cells were transfected using the DEAE-dextran method according to Choi and Engel (25) and the P388 D1 cells according to Queen and Baltimore (26). All cells were harvested 2 days after transfection and assayed for CAT activity (27). Diagnostic cotransfection with a control plasmid showed the reproducibility of the transfections, therefore CAT activities achieved did not have to be corrected.

RESULTS

Mouse M-lysozyme gene domain acquires a myeloid-specific and differentiation dependent chromatin configuration

The M-lysozyme gene is a marker gene of myeloid differentiation and offers attractive features for studying the role of chromatin in cell type and cell stage specific activation of eukaryotic genes. In order to study differentiation specific changes in chromatin structure, we performed DNase I hypersensitive site analysis in various cell lines reflecting different stages of myeloid differentiation. The RMB1 cell line represents a very early myeloid precursor cell line, Wehi 3b is a more mature but still not fully differentiated myelo-monocytic leukaemia cell line, whereas the J774.16 cell line consists of macrophages and represents the most mature stage in myeloid differentiation (10). The transcriptional activity of the M-lysozyme gene as determined by RNA- and protein analysis parallels these stages of differentiation (2). A major purpose of this study was to detect changes in the pattern of DNase I HSs during the developmental activation of this gene.

Nuclei isolated from RMB 1, Wehi 3b, J774.16 and as a control from a lysozyme non-expressing hematopoietic cell (T-cell line EL 4) were digested with increasing concentrations of DNase I. The DNA was purified and the nuclease hypersensitive sites in the chromatin were mapped by indirect end-labeling of restriction enzyme-digested DNA segments (11). For the analysis

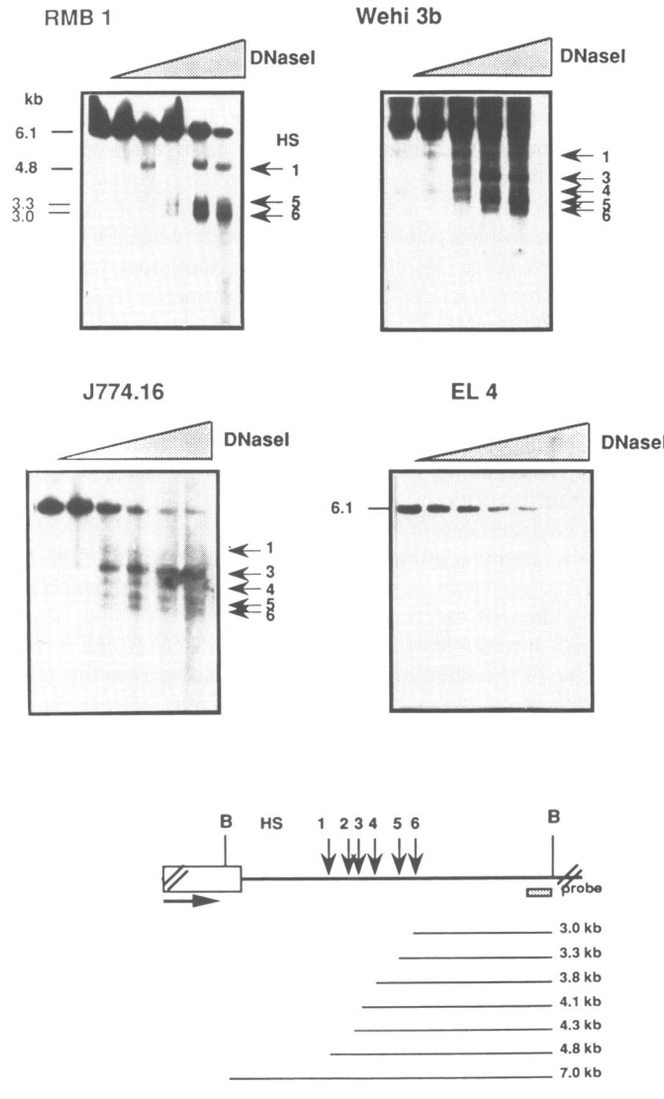


Fig. 2. Mapping of DNaseI HSs in the 3'-region of the gene. A 20 μg sample of each DNA shown in Fig. 1 was cut with Bam HI, electrophoresed in a 1% agarose gel, blotted and indirectly endlabeled with the probe shown in the diagramm below. HSs are indicated by arrows. The diagram shows the positions of DNaseI HS 1 to 6 (vertical arrows). See legend to Figure1 for an explanation of symbols, position of HS2 is determined from Fig. 5.

of 5′ and intragenic regions the DNA was digested with Hind III (HS 10−16) and of the 3′-region the DNA was digested with Bam HI (HS1−6). As shown in Fig. 1 there is a complex array of 7 DNaseI HS (HS 10−16) in the 5′-region of the gene, which are established differentially in the myeloid cell lines. The HS 15 located at −400 bp from the transcriptional start site is very prominent and can be found in all tested myeloid cell lines, whereas the HS 16 at + 300 bp in the first intron and HS 13 (−1.6kb) are specific for the mature macrophage cell line J774.16. However the less sensitive sites HS 14 (−1.2 kb) and HS 12 (−2 kb) are specific for the earlier stages of myeloid differentiation, as they only appear in the chromatin of the RMB1 and Wehi 3b cells. HS 10 (−3.8kb) could only be found in the RMB 1 cells, probably being specific for the very early myeloid precursor stage.

We next examined the nuclease hypersensitivity in the 3′-region of the gene (Fig. 2). A prominent and rather complex pattern of HSs is located in the region +5.4 kb to + 7.3 kb. This region is of particular interest, since it co-localizes with a DNA sequence specific for the M-lysozyme gene domain. Since lysozyme M- and P-genes are products of a recent gene duplication event (1) the majority of DNA fragments are common to both P and M domains. In this M-specific region are 6 potential DNaseI HSs, the pattern of which changes in the different myeloid cell lines. The HSs 1, 5 and 6 are well established in the myeloid cell line RMB1, they are also found in the chromatin of the more differentiated cell line Wehi 3b, although with reduced intensities for HS 1 and HS 6. Wehi 3b shows two additional HS, HS 3 and HS 4. This tendency is continued in the mature macrophage cell line J774.16. HS 3, as compared to the other HSs is very strong at this stage. Therefore the DNaseI sensitivity of HS 3 seems to increase during myeloid differentiation. Interestingly the chromatin of the M-lysozyme gene domain is completely inaccessible for DNaseI in the lysozyme non-expressing T-cell line El 4, whereas the overall sensitivity to DNase I seems to increase during differentiation of myeloid cells although this has not been analyzed carefully. Assuming that the cell line specific HS patterns found upstream, within and downstream of the M-gene reflect regular *in vivo* stages in myeloid differentiation the following changes can be summarized: HS 10, 12 and 14 are specific for the immature stage, whereas HS 16, 3 and 4 only occur at the fully mature macrophage stage. However at this point it could not be excluded that particular HSs may be specific for a particular cell line rather than being specific for a defined stage in differentiation of myeloid cells. Therefore we analyzed hematopoietic stem cell lines before, during and after differentiation to macrophages. If the HS patterns determined in the cell lines described above do reflect specific stages of myeloid differentiation in general, then the same HS patterns should be visible during *in vitro* differentiation of stem cell lines. In addition it was of interest to study early events, during which HSs may be established. Since the myeloid precursor cell line RMB 1 already displays hypersensitivity in chromatin, whereas the lymphoid cell is completely insensitive in this region, we were interested in analyzing stages in hematopoiesis earlier than that which the RMB1 cells represent.

Differentiation of two multipotent hematopoietic stem cell lines towards mature myeloid cells

Both interleukin-3 dependent cell lines FDCP-A4 and C2GM are continuosly growing multipotential hematopoietic stem cell lines (9). In appropriate culture conditions (either in the presence of growth and serum factors or in association with marrow stromal cells), the cells can be induced to undergo multilineage differentiation and development leading to the production of mature blood cells and clonal extinction. In order to determine the conditions favouring myeloid differentiation to mature macrophages, the following growth factors were tested in various combinations and concentrations: rIl3, rG-CSF, rGM-CSF and L-cell-conditioned medium as a source of M-CSF. FDCP-A4 and C2GM cells were maintained under these respective conditions for 7 or 8 days. The number of myeloid precursor cells and mature cells was determined by morphological analysis, and this allowed us to ascertain the optimal conditions (1.5 u/ml rIl3, 1000 u/ml rG-CSF, 50 u/ml rGM-CSF, 15% L-cell-conditioned medium) for macrophage/granulocyte differentiation of both cell lines. After 7 to 8 days culture in these conditions about 55% to 65% of the cells show the characteristics of mature macrophages (27% to 35% were mature neutrophilic granulocytes, the others represented earlier myeloid maturation stages). In addition lysozyme protein and mRNA assays were performed. Equal amounts of RNA were seperated on 1% agarose 2.2M formaldehyde denaturing gel, blotted and hybridized with a M-lysozyme cDNA probe. A typical northern blot of mRNA preparations from day 0 (multipotent stage) of both cell lines, and from cells at various times of culture in the differentiation medium, is shown in Fig. 3a. At the multipotent stage there is a very low level of lysozyme mRNA detectable. However, by day 4 of culture under differentiation conditions, levels of lysozyme mRNA were very high, and only a marginal

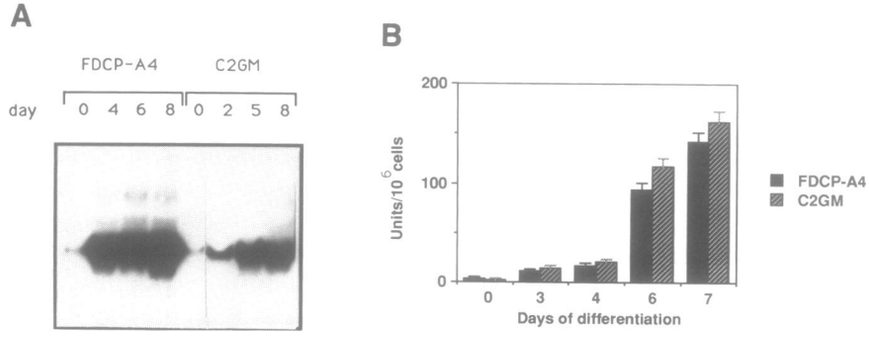


Fig. 3. Lysozyme gene activity during differentiation. **(A)** Northern analysis of 20 μg total RNA at various days of differentiation of the FDCP-A4 and C2GM cells. After electrophoresis in a 1% formaldehyde agarose gel and blotting, hybridization was performed with a M-lysozyme cDNA probe. **(B)** Measurement of lysozyme enzyme activity (units/10^6 cells) at different days of differentiation of both hematopoietic stem cell lines.

further increase was detected by day 6 and 8. Similiar results are obtained during differentiation of the C2GM cells. There is already an increase of lysozyme mRNA amount observed at day 2 with the maximal amount seen at day 5. In general the mature C2GM cells seem to produce less lysozyme mRNA than the mature FDCP-A4 cells.

In order to determine the lysozyme protein production during myeloid differentiation the lysozyme activity was assayed turbidometrically in cell extracts of various differentiation stages (Fig. 3b). At day 0 there is a very low level of 4 u/10^6 cells in the case of the FDCP-A4 cells and 3u/10^6 cells in the case of the C2GM cells measurable. Up to day 4 the lysozyme protein amount increases slowly, but by day 6 there is a major increase detectable. At day 7 the lysozyme activity amounts to 140 u/10^6 cells (FDCP-A4) and 160 u/10^6 cells (C2GM).

Chromatin analysis during hematopoiesis from the stem cell to the mature myeloid stage

The chromatin of the two hematopoietic stem cell lines FDCP-A4 and C2GM was analyzed before induction of differentiation (i.e. as multipotent stem cells undergoing self renewal), and also at day 3 and 7 post-induction of differentiation (for the FDCP-A4 cells) and day 8 (for the C24M cells). The HSs of the 5′-region of the M-lysozyme gene in these cells are shown in Fig. 4. The HS pattern at the multipotent stage of both cell lines exactly reflects the situation as observed in the RMB 1 cell line, with the exception that the chromatin of the C2GM cells already demonstrates very weak hypersensitivity at site 16 and no obvious sites 12 and 14. Following differentiation HS 12 and 14 disappear and HS 16 appears during differentiation in the FDCP-A 4 cell line.

The pattern and the sensitivities of the HSs in the 3′-region (Fig.5) also resemble the ones observed in the myeloid cell lines. HS1, 5 and 6 are present in all stages of differentiation with slight differences in their sensitivity. HS 5 and 6 lose sensitivity during the differentiation. In contrast the chromatin in between HS 1 and 5 becomes more and more nuclease sensitive, such that at day 3 only a faint band indicative of HS 3 is visible, which becomes very strong by day 7 of FDCP-A4 and day 8 of C2GM cell lines. In addition HS 3 is flanked at the most mature differentiation stages by HS 2 and HS 4.

Functional analysis of sequences in the 3′HS cluster identifies a strong enhancing element

In an attempt to correlate DNase hypersensitivity to regulatory function, DNA fragments carrying various combinations of the M-lysozyme HSs were inserted upstream from the tk promoter driving the CAT reporter gene, and promoter function was assayed following transient transfection of the DNA into both lysozyme expressing and non-expressing cell lines. In addition, constructs in which the CAT gene was fused directly to the LysM promoter and upstream regions (covering a total at 20 kb of the lysozyme gene region) were also assayed by transient transfection.

Since the J774.16 and EL4 cells were unsuitable due to unsatisfactory transfection efficiencies, all constructs were tested in the myeloid cell lines P388D1 (mature macrophages), RMB1 (myeloblasts), and in the fibroblastic cell line NIH3T3 as a negative control.

None of the constructs containing either M lysozyme promoter and upstream regions or combinations of 5′ HS sites (HS 10−15) inserted upstream from the tk promoter showed strong effects in any of the cell lines tested. In contrast, some combinations of the 3′ HSs were found to affect CAT expression (Fig. 6). The construct pHS 1 tkCAT (in both orientations) lowers the transcriptional rate weakly, whereas pHS 2 tkCAT and pHS 5/6 tkCAT in sense orientations showed a more or less neutral effect in all tested cell lines. In contrast, pHS 3 tkCAT, which includes

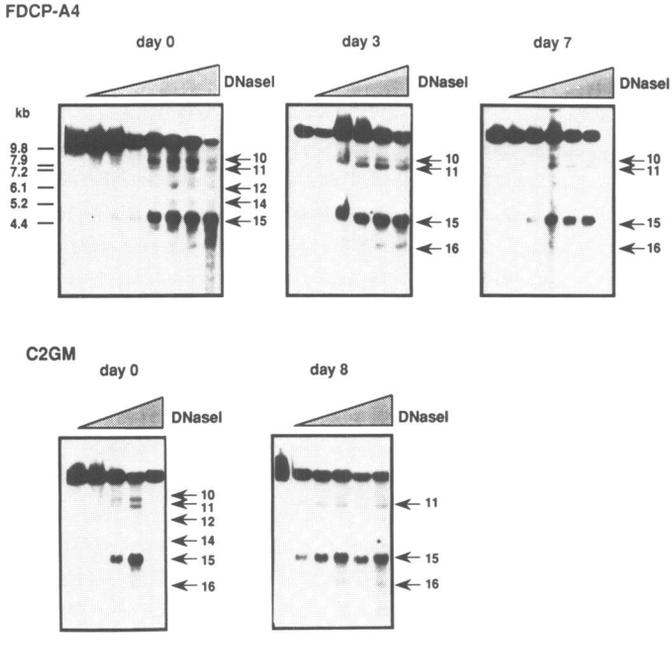


Fig. 4. DNaseI HS mapping of different days of differentiation of both hematopoietic stem cell lines in the 5′and intragenic region. HSs are marked by arrows. Nuclei were DNaseI digested and analyzed as desribed in the legend to Fig.1.

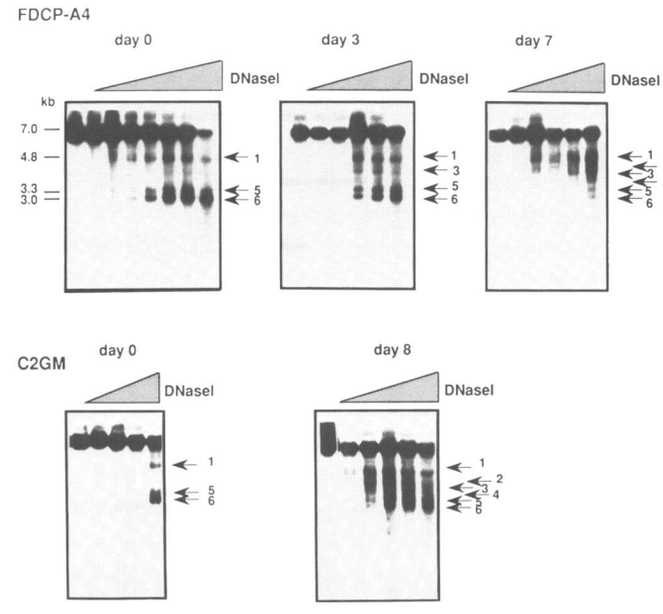


Fig. 5. DNaseI mapping of the 3′region of the gene in different stages of differentiation of both hematopoietic stem cell lines. Analysis as in the legend to Fig. 2.

the region of the differentiation specific HS 3 shows a strong positive effect, which is most pronounced in RMB1 cells (similar in magnitude as the SV40 viral enhancer). This enhancing effect is reduced in antisense orientation. A very weak positive effect is detectable with the construct pHS 4 tkCAT in sense orientation. The DNA spanning the regions of the differentiation specific HSs 2, 3 and 4 is contained in the plasmid pHS 2/4 tkCAT. This is neutral in NIH 3T3 cells, whereas a weak enhancing effect could be determined in RMB1 and P388D1 cells with both orientations.

Thus, while HSs 1,2,4,5 and 6 appear to have little or no independent effects on the promoter function, HS3 results in a strong increase in transcription. The effect of HS3 appears to be largely context dependent, since it is reduced both by reversal of the orientation of HS3 with respect to the promoter and by combining HS3 with the flanking sites 2 and 4.

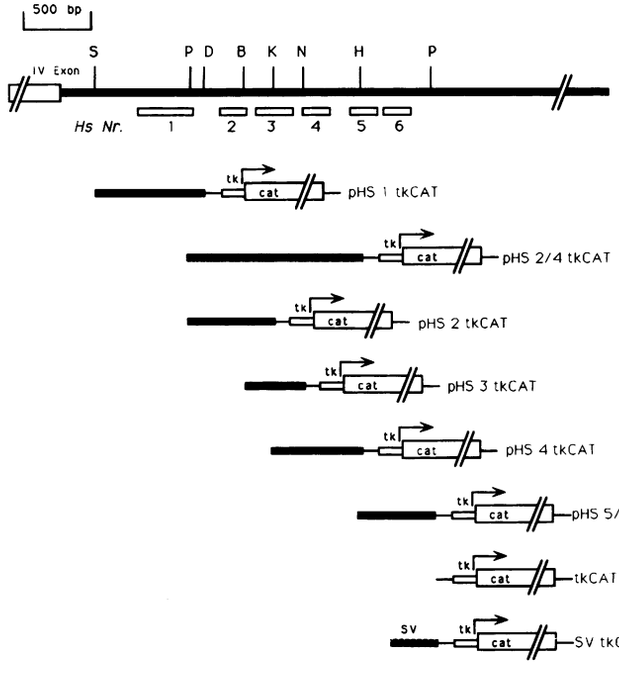
DISCUSSION

Myeloid- and differentiation specific HSs were detected in the mouse M-lysozyme gene domain

We performed DNase I hypersensitive site analysis in various myeloid cell lines and in a T-cell line, choosing myeloid cell lines which can be 'ordered' according to their maturity (10): the precursor cell line RMB1, the immature macrophage cell line Wehi3b and the mature macrophage cell line J774.16. The lysozyme gene transcriptional activity and lysozyme protein amounts parallels these developmental stages (2). No transcriptional activity has been detected in lymphoid cells such as EL4 (12). A complex pattern of many HSs specific for different stages of differentiation was detected (Fig.7). Some HSs (for example HS15,11,1 and the pair HS 5 and 6) appear in the chromatin of each of above myeloid cell lines, whereas some HSs are specific for a distinct differentiation stage. The HS 16 which is localized in the first intron could only be seen in the chromatin of J774.16 cells. The appearence of two other HS sites (3 and 4) was also correlated to increasing maturity. In contrast HS 10, 12 and 14 are specific for the immature stage. In a lympoid cell line (EL4) which does not express lysozyme no HS could be detected in the M gene region, so that all HS observed can be considered to be tissue specific. Interestingly, a complex pattern of HSs appear before the marked increase in levels of lysozyme mRNA. This genearal feature is observed in a variety of genes and shows that HSs are a nessecary requirement for gene expression (see for review: 6). For instance, the chromatin of the chicken lysozyme gene shows HSs in immature oviduct, where the gene is silent (13, see also for review: 14). Similarly, the HSs of the human globin locus are formed before the onset of globin transcription (15).

Changes in chromatin structure during hematopoiesis

The two multipotent hematopoietic cell lines FDCP-A 4 and C2GM can be induced in appropriate culture conditions (in the presence of growth and serum factors) to undergo multilineage differentiation leading to the production of mature blood cells and clonal extinction. When cultured in the presence of Il3, G-CSF, GM-CSF and M-CSF these cells differentiate exclusively into the myeloid direction. The production of M-lysozyme mRNA and protein parallels this differentiation. Remarkably the



Relative CAT-activity

	NIH 3T3	+/-	RMB 1	+/-	P388 D1	+/-
s	0.7	0.2	0.8	0.4	0.5	0.3
a	0.2	0.1	0.6	0.1	0.4	0.1
s	0.8	0.4	2.6	0.1	3.0	0.1
a	1.3	0.8	3.6	0.4	3.5	0.1
s	2.2	0.7	1.5	0.4	1.8	0.5
a	n.d.		n.d.		n.d.	
s	18.0	1.5	36.2	11.1	15.5	2.2
a	2.7	0.6	6.0	0.6	3.2	0.9
s	1.5	0.6	2.6	0.4	2.5	0.6
a	0.3	0.1	1.8	0.2	1.7	0.6
s	0.8	0.2	1.0	0.2	1.1	0.1
	1.0	0.2	1.0	0.3	1.0	0.1
	n.d.		28.7	3.3	26.8	3.5

Fig. 6. Transient transfection analysis of the 3'-region of the M-lysozyme gene. The last exon (IV) and some restriction sites are indicated: S = SpeI, D = NdeI, B = BglII, K = KpnI, N = NheI, H = HindIII, P = PvuII. The regions of the DNaseI hypersensitivity are marked by boxes. A series of fragments was fused to the CAT gene, driven by the Herpes simplex thymidine kinase (tk) promoter. NIH 3T3 (fibroblasts), RMB 1 (myeloblasts) and P388 D1 cells (mature macrophages) were transfected as described in Materials and Methods. The table on the right represents the relative CAT-activities of the respective constructs relative to the activity seen with tkCAT. All constructs were tested in at least three independent experiments in each cell line. Standard deviations are given in italic numbers (*s* = sense orientation, *a* = antisense orientation).

multipotent stage of both cell lines reveal exactly the same pattern of HSs as observed in the RMB1 cells (Fig. 7). Of the HSs normally associated with more mature stages, only HS 16 is already detectable as a faint band at day 0 of the C2GM cells. This result suggests that the chromatin domain already contains hypersensitive sites at the multipotent stage prior to lineage commitment. Although unlikely, an alternative explanation might be that the undifferentiated stem cell lines consist of a heterogenous population of precursors for all potential progeny cell types, which are overgrown by the myeloid lineage upon differentiation. Under these circumstances, non-macrophage/granulocyte precursor cells would not show HSs in the lysozyme domain, but outgrowth would result in a large increase in the overall fraction of cells harboring lysozyme HSs. Such a change is not found; although individual HSs do change their intensities, the overall hypersensitivity in the lysozyme gene region does not change and must therefore be present in multipotential precursor cells. Similar observations have been made for the Ig heavy chain gene enhancer, in which a HS is detectable in B cell precursors and T cells as well as in myeloid-lymphoid precursor cells, but upon myeloid differentiation it adopts a closed chromatin configuration (16). Similar results have also been obtained for the MHC class I H2-D locus (17) and for the human globin genes (18). The M-lysozyme system differs in respect to the chromatin structure of the globin system, since HSs in the globin domain were detected in non-erythroid and even in non-hematopoietic cells, whereas the chromatin of the M-lysozyme gene domain is absolutely nuclease insensitive in the lymphoid lineage.

Upon myeloid differentiation of both hematopoietic progenitor cell lines HSs patterns were found, which correlate exactly to those found in the later myeloid cell lines (Fig.7). A strong effect of differentiation can be noticed in the 3'-region of the gene. HS 1, 5 and 6 are present at all stages with slight differences in their intensities, with HS 5 and 6 becoming weaker during differentiation. In contrast, the region around HS 3 becomes increasingly more DNaseI accessible: HS 3 is first detectable at day 3 of differentiation and becomes more sensitive later on. HS 3 appearence is accompanied by two other HSs nearby, HS 2 and 4. This differentiation specific HS region is interesting, since it resides in a M-gene specific region, whereas the majority of DNA sequences of the M-gene are found in the P-gene as well (1). The kinetic of the HS 3 appearence parallels the kinetic of gene activation.

Functional relevance of the 3'HS cluster

It is known that sites hypersensitive to DNase I often reside within *cis*-acting DNA sequences that are crucial for gene expression. Therefore, DNA sequences spanning many or single HSs were tested in transfection assays. We found that the 5' region is not sufficient for expression in macrophages. This can be compared to the fact that the downstream copy (M-gene) of the gene retained myeloid specificity after the gene duplication (1) indicating that there was (and is) no requirement for sequences further upstream than the duplication border (−1.7 kb). This is also consistent with a requirement for downstream regulatory elements. One group of HSs with a strong correlation to expression indeed maps downstream, and coincides exactly with the M-specific fragment (1). This region, which is a good candidate for a regulatory element does show enhancer activity. One subunit of this region (HS3) shows strong enhancer activity, but no apparent specificity. The strongest effect of 30 to 40-fold CAT-induction in sense orientation was found in the RMB1 cells, in which the endogenous HS is totally absent. This suggests two posssibilities: Firstly, transcription factors responsible for the enhancement are present in an active form in M-lysozyme expressing and non-expressing cells (unlike e.g. NFκB which is present in an inactive form; 19); secondly, these factors can mediate transcriptional activation when the *cis*-acting DNA sequence is present in an accessible form as is the case in transient transfections. That this effect is

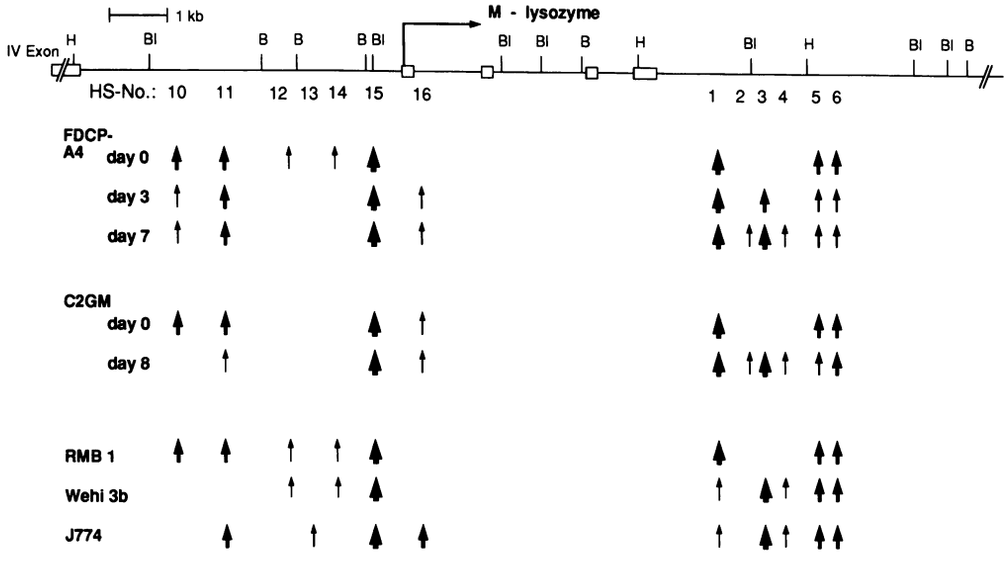


Fig. 7. DNaseI HSs in the M-lysozyme gene. The M-lysozyme gene from −4.6 to +10.2 kb is depicted. The start site and direction of transcription are indicated by an horizontal arrow. The exons are indicated by black boxes. Relevant restriction sites are marked: B = Bam HI, Bl = BglII, H = HindIII. The various DNaseI HSs are indicated by vertical arrows and are numbered from 10 to 16 (intragenic and 5'-region) and from 1 to 6 (3'-region). The degree of DNaseI sensitivity of each HS is represented by the size of the arrows.

stronger in sense than in antisense orientation might be due to the local spacing of binding sites in particular constructs. Similar effects have been obseved with other transfected *cis*-acting elements, such as that of the human α1-antitrypsin gene (20). The fact that transcriptional activation of the construct pHS 2/4 tkCAT, which harbors the region of HS 3, is weaker compared to pHS 3 tkCAT could mean that additional negatively acting sequences are located on this fragment.

Thus, the evidence from the transfection experiments suggests that the tissue specific function of some regulatory elements can not be due solely to the tissue specific distribution of their corresponding transcription factors, but may involve cell type-specific alterations at the chromatin level. The consequences of DNA modification for the binding of a nuclear protein to the HS3 region are analyzed in the accompanying manuscript.

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REFERENCES

1. Cross, M., and Renkawitz, R. (1990) *EMBO J*, **9,** 1283–1288.
2. Cross, M., Mangelsdorf, I., Wedel,, A. and Renkawitz, R. (1988) *Proc. Natl. Acad. Sci. USA*, 85, 6232–6236.
3. Weintraub, H., and Groudine, M. (1976) *Science*, **193,** 848–856.
4. Elgin, S. C. R. (1988) *J.Biol.Chem.*, 263, 19259–19262.
5. Elgin, S. C. R., (1990) *Curr.Op.Cell.Biol.*, **2,** 437–445.
6. Gross, D. S., and Garrard, W. T. (1988) *Annu.Rev.Biochem.*, **57,** 159.197.
7. Weisbrod, S. (1982) *Nature*, **297,** 289–295.
8. Heyworth, C. M., Dexter, T. M., Kn., O., and Whetton, A. D. (1990) *Growth factors*, **2,** 197–211.
9. Spooncer, E., Heyworth, C. M., Dunn, A., and Dexter, T. M. (1986) *Differentiation*, **31,** 111–118.
10. Leenen, P. J. M., Jansen, M. A. C., and van Ewijk, W. (1986) *Differentiation*, **32,** 157–164.
11. Wu, C. (1980) *Nature*, **286,** 854–860.
12. Ralph, P., Moore, M. A. S., and Nilsson, K. (1976) *J. Exp. Med.*, **143,** 1528–1533.
13. Fritton, H. P., Igo-Kemenes, T., Nowack, J., Strech-Jurk, U., Theisen, M., and Sippel, A. E. (1984) *Nature*, **311,** 163–165.
14. Sippel, A. E., and Renkawitz, R. (1989) In Renkawitz, R. (ed.) tissue specific gene expression. VCH Verlagsgesellschaft, Weinheim, pp 185–198.
15. Forrester, W. C., Thompson, C., Elder, J. T., and Groudine, M. (1986) *Proc.Natl.Acad.Sci., USA* **83,** 1359–1363.
16. Ford, A. M., Watt, S. M., Furley, A. J. W., Molgaard, H. V., and Greaves, M. F. (1988) *EMBO J.*, **7,** 2393–2399.
17. Maschek, U., Pülm, W., and Hämmerling, G. J. (1989) *EMBO J.*, **8,** 2297–2304.
18. Dhar, V., Nandi, A., Schildkraut, C. L., and Skoultchi, A. (1990) *Mol.Cell.Biol.*, **10,** 4324–4333.
19. Baeuerle, P. A., and Baltimore, D. (1988). *Cell* , **53**, 211–217.
20. Ciliberto, G., Dente,L., and Cortese, R. (1985) *Cell,*. **41,** 531–540.
21. Stanley, E. R. and Heard, P. M. (1977) *J. Biol. Chem.*, **252**, 4305–4315.
22. Feinberg, A. P., and Vogelstein, B. (1983) *Anal.Bioch.*, **132,** 6–13.
23. Maniatis, T., Fritsch, E. F., and Sambrook, E. F. (1982) Cold Spring Harbor Laboratory, NewYork.
24. Steiner, C., and Kaltschmidt, C. (1989) *TIG* , **5,** 138.
25. Choi, O.-R. B., and Engel, J. D. (1988) *Cell* , **55,** 17–26.
26. Queen, C., and Baltimore, D. (1983) *Cell*, **33,** 741–748.
27. Gorman, C. M., Moffat,L. F., and Howard, B. H. (1982) *Mol.Cell.Biol.*, **2,** 1044–1051.