The involvement of demethylation in the myeloid-specific function of the mouse M lysozyme gene downstream enhancer

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ABSTRACT

Lysozyme gene expression is a specific marker for the macrophage/granulocyte lineage of hematopoietic differentiation in mammals, its expression being gradually increased during maturation. Analysis of the mechanisms regulating mouse M lysozyme gene expression during myeloid differentiation revealed a complicated pattern of DNase I hypersensitive sites (HS sites) within the flanking regions of the gene. The HS-3 site, located in the 3'-flanking region of the gene, overlapped with an enhancer element, which is the only strong enhancer identified in the vicinity of the gene. We demonstrate a positive correlation between undermethylation of the entire 3'-flanking region, the appearance of the HS-3 site, and M lysozyme gene expression during in vitro differentiation of hematopoietic stem cells. We furthermore show that methylation of a single CpG site within the enhancer core element, only observed in immature macrophage cells in vivo, is sufficient to inhibit nuclear factor binding to this element in vitro and to inhibit its trans-activation potential in DNA transfection experiments.

INTRODUCTION

The regulation of tissue-specific genes is of key interest in molecular biology. The establishment and maintenance of specific gene expression in mammalian cells may involve a number of strategies including the presence or absence of specific factors which regulate transcriptional activity by direct interaction with the DNA, changes in chromatin structure, and DNA modifications. For eukaryotic DNA, the best described modification is cytosine methylation, which is almost exclusively restricted to the dinucleotide CpG in vertebrates (1). Many studies have shown an inverse correlation between the extent of DNA methylation and the level of gene expression. Specific CpG methylation is apparently involved in inactivation of gene expression and seems to play a role in tissue and developmental-stage specific gene expression (for reviews, see 2, 3). The effects of DNA methylation on the (possibly multistep) process of gene activation during differentiation can not be examined in many systems due to the availability of only mature cell types. The study of myeloid differentiation systems can largely overcome this limitation, since much of the pathway can be studied step by step in vivo and in vitro. This is especially true of the mouse system due to the availability of a number of mouse myeloid cell lines representing different stages of myeloid differentiation (4). Lysozyme gene expression is a specific marker for the macrophage/granulocyte lineage of hematopoietic differentiation in mammals, its expression being gradually increased during maturation (5). The mouse M lysozyme gene is one of two lysozyme genes generated by a recent gene duplication event, and retains the ancestral myeloid expression pattern (6). The gradual increase in expression during macrophage/granulocyte maturation is evident from analysis of M lysozyme gene expression in stage-specific cell lines (5), and in hematopoietic stem cells undergoing differentiation in vitro (Möllers et al., accompanying manuscript).

Analysis of the mechanisms regulating M lysozyme gene expression during myeloid differentiation revealed a complicated pattern of DNase I hypersensitive sites (HS sites), which exhibited well defined changes during in vitro differentiation of stem cells to macrophages (Möllers et al., accompanying manuscript). One of these HS sites, located downstream of the gene, was found to correlate to an enhancer element which seems to be the only strong enhancer in the vicinity of the gene. However, the enhancer did not show tissue-specific activity in transfection assays.

In this paper, we demonstrate that undermethylation of the downstream region correlates strongly both to the presence of HS3 and the expression of the lysozyme gene. Furthermore, we identify an enhancer binding activity in nuclear extracts from both lysozyme expressing and non expressing cells, and show that binding is inhibited by methylation of a single CpG site in the enhancer core element. This modification blocks the function of the enhancer element in transient transfection experiments, and is detectable in endogenous DNA only in immature or non-

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myeloid cells. We postulate that cell type specific demethylation is involved in the tissue specific expression of the mouse lysozyme M gene.

MATERIALS AND METHODS

Plasmids and oligonucleotides

Plasmids were constructed by standard techniques (22). Sense and antisense orientations are indicated by 's' and 'a', respectively. All plasmid DNA was isolated and purified by a Trition X100 lysis followed by centrifugation in a CsCl gradient. All M lysozyme gene fragments were isolated from subclones of cosmid clone cosmL.g14 (6).

The plasmids pH52/4tkCAT (+5.8/+6.6 kb; BglII/HindIII fragment), pH53tkCAT (+5.8/+6.2 kb; BglII/NheI fragment), pH53.1tkCAT (+5.8/+6.0 kb; BglII/KpnI fragment), and pH53.2tkCAT (+6.0/+6.2 kb; KpnI/NheI fragment) were created by inserting the filled-in fragments into the filled-in BamHI site of ptkCATbH/N (23). Orientation and correct insertion of the fragments was determined by restriction mapping. The synthetic double-stranded MLDE oligonucleotide (5'-CTATAGGTAGGGACTCC-3') was inserted into the filled-in SalI site of ptkCATbH/N; orientations were determined byideoxy-sequencing using the Sequenase-kit from USB. For pSVtkCAT see (24).

For band shift assays the MLDE and MLDE mut. (5'-CTATAGGTAGGGACTCC-3'; +6.1/+6.15 kb) was inserted into the filled-in Sall site of ptkCATbH/N; orientations were determined byideoxy-sequencing using the Sequenase-kit from USB. For pSVtkCAT see (24).

The in vitro methylation of plasmid DNA or MLDE oligonucleotide was performed using HpaII methylase (Pharmacia) according to the instructions of the supplier. Complete methylation was verified by digesting the DNA with an excess (20 μg) ofHpaII restriction enzyme. Unmethylated control DNA's were prepared identically without addition of methylase. Prior to transfection, the methylated and mock-methylated DNA's were phenol-extracted and ethanol-precipitated.

Cell culture, transfections and CAT assays

Mouse L cells, NIH3T3 fibroblasts, and mouse myelocytes RMB1 (4) were grown in Dulbecco's modified Eagle's medium (DMEM; Biochrom) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 U/ml streptomycin (Pen/Strep). Mouse macrophages P388D1 (4) were grown in a 1:1 mixture of DMEM and RPMI 1640 medium (Gibco) supplemented with 10% FCS and pen/strep, and havested for subcultivation in EDTA instead of trypsin. Mouse macrophages J774-1.16 (4) were cultivated in DMEM containing 10% horse serum, 5% NCTC 109 medium (Sigma), 1% non-essential amino acids (100×, Gibco), and 1% L glutamine (100×, Gibco). FDCP-A4 cells were cultivated and differentiated in vitro as described in Möllers et al. (accompanying manuscript).

For demethylation, cells were grown for 72 hours in the presence of 6 μM (NIH3T3 cells) or 4 μM (all other cells) 5'-azacytidine (Sigma).

DNA transfections of NIH3T3 fibroblasts were performed as described by Steiner and Kaltschmidt (29). P388D1 macrophages were transfected using diethylaminoethyl-dextran (DEAE-dextran; 30). RMB1 myelocytes and mouse L cells were transfected in suspension by a modified DEAE-dextran method (31). In all cases 1 pMol of plasmid DNA was used per dish. All transfections were carried out in triplicate and confirmed in a second series of tests.

CAT assays were performed as described by Gorman et al. (32).

Southern blot hybridizations

Genomic DNA of mouse cell lines and of in vitro differentiated FDCP-A4 cells was prepared by SDS/protease K treatment according to Ausubel et al. (22). High molecular weight DNA was digested with HindIII, Mspl and HpaII (Boehringer, Mannheim) at 5 μg DNA for 16 hours at 37°C and separated on 1.8% agarose gels. Southern blotting and hybridizations were performed as described by Cross and Renkewitz (6). For hybridization probe the BglII/HindIII-M lysozyme gene fragment (+5.8/+6.6 kb) was isolated from a 1% agarose gel by cutting out the corresponding band, freezing the agarose in liquid nitrogen, and centrifuging through glass wool.

Northern blot hybridizations

Total RNA from 5'-azacytidine treated or untreated control cells was isolated using guanidinium isothiocyanate lysis and centrifugation through a CsCl cushion (33). Northern blotting was performed as described by Cross et al. (5). Hybridizations using a 32P-labeled M lysozyme cDNA probe (5) were performed as for the Southern blot hybridizations. The Drosophila β1-tubulin cDNA probe was kindly provided by D. Buttgereit and R. Renkewitz-Pohl. Hybridizations with this probe were performed at 50°C, with washing at 45°C.

Fig. 1. Influence of demethylation on the expression of the endogenous M lysozyme gene in fibroblasts (NIH3T3), myelocytes (RMB1), and mature macrophages (P388D1, J774). Total RNA was purified from 5'-azacytidine treated (+) or untreated (−) cells, and 40 μg (NIH3T3) or 20 μg (all other cells) of RNA were used for Northern blot analysis. The 1.4 kb M lysozyme transcript is marked by an arrow. Upper panel: Hybridization with the M lysozyme cDNA probe. Lower panel: Hybridization with the tubulin probe.
Band shift and footprint assays
Nuclear protein extracts of RMB1 and P388D1 cells used for band shift and footprint assays were isolated according to Schreiber et al. (34). Band shift and footprint assays were carried out as described by Altschmied et al. (35), with the exceptions that 5 µg of nuclear extracts were used for each lane in case of the band shift assays and 75 µg for the footprint reactions. The blunt-ended oligonucleotides were end-labeled using polynucleotide kinase. Probes for footprinting were prepared from Eco RI cut plasmids pHS 3 tkCAT and pHS 3/2 tkCAT and the 5'-overhangs were filled in with Klenow enzyme and [α-32P]dATP. The labelled DNA was digested with Xba I and fragments were purified by gel electrophoresis.

RESULTS
Demethylation leads to activation of the M lysozyme gene in myelocytes
Previous results revealed a contradiction between the tissue specific expression of the M lysozyme gene and the differentiation specificity of the HS-3 site with the lack of cell type specificity of the mouse M lysozyme downstream enhancer (MLDE) in transient transfection experiments. In addition, DNase I footprinting experiments with extracts from M lysozyme expressing and non-expressing cells revealed no difference in the binding of nuclear factors to the MLDE (data not shown). A possible explanation for this apparent contradiction might be, that the specificity is not conferred by the DNA binding proteins, but rather by a modification of the DNA. Since inactivation of genes in developing cells by DNA hypermethylation has been previously reported (2), we considered DNA methylation as a possible mechanism for inactivation of the endogenous M lysozyme gene in immature macrophages and non-expressing cells.

Treatment of cells with 5'-azacytidine, a nonspecific inhibitor of DNA methylation (7), has been shown to activate expression of genes regulated by DNA methylation (2). Therefore, such treatment should give an indication of whether the M lysozyme gene is regulated by methylation. NIH3T3, RMB1, P388D1, and J774 cells were treated with 5'-azacytidine, genomic DNA was purified and demethylation was verified by Mspl or HpalI digestion and Southern blotting (data not shown). Total RNA was purified in parallel from treated and untreated control cells, blotted, and hybridized with an M lysozyme cDNA probe. Fig. 1 shows that in the absence of 5'-azacytidine the expression rates of the endogenous gene are typical for the analyzed cell types; no detectable expression in fibroblasts, small mRNA amounts in immature cells, and intermediate to high expression in both macrophage cell lines. DNA demethylation after 5'-azacytidine treatment leads to a strong activation of the M lysozyme gene in RMB1 myelocytes, and a slight increase of expression in P388D1 macrophages, whereas the high level of expression in J774 cells is not further increased (Fig.1). In NIH3T3 fibroblasts, a totally unrelated cell type, it is not possible to activate the M lysozyme gene solely by DNA demethylation: although double the amount of RNA from NIH3T3 cells is used than from the other cell types, no lysozyme mRNA is detectable after 5'-azacytidine treatment (Fig.1). The same RNA blot was stripped and rehybridized with a tubulin probe to guarantee that the observed effects are not due to variation in RNA amounts (Fig.1).

Correlation of cell type specific DNA demethylation with the level of M lysozyme expression and the presence of a DNaseI hypersensitive site
Since 5'-azacytidine treatment indicated that the M lysozyme gene may be regulated by DNA methylation, we analyzed the methylation pattern of the downstream enhancer region in

![Fig. 2. Mspl/HpalI restriction map of the M lysozyme gene downstream region. Map of the M lysozyme gene (upper part): exons are boxed and marked with roman numerals, the vertical arrows represent DNase I hypersensitive sites 1 to 6 (Müllers et al., accompanying manuscript). Downstream region (lower part): restriction sites are indicated (H=Mspl/HpalI, individual sites are numbered; K=KpalI, B=BglIII, H=HindIII); the expected fragment sizes for HindIII, Mspl/HpalI digestion, and incomplete HpalI or HpalI/KpalI digestion (as found in NIH3T3 cells, Fig.3), are given. The BglII/HindIII fragment used as hybridization probe is shown below the map, the transactivating clones pHS3, pHS3.2 and the MLDE oligonucleotide are represented by boxes.](image)

![Fig. 3. Comparison of methylation patterns of the M lysozyme downstream region in fibroblasts, myelocytes, and mature macrophages. Genomic DNA (20 µg for each lane) from different cell lines digested with Mspl, HpalI (for RMB1 cells after predigestion with HindIII), or HpalI/KpalI was hybridized to the BglII/HindIII fragment (Fig.2). The fragments originating from complete Mspl or HindIII digestion, or from incomplete HpalI or HpalI/KpalI digestion are marked by arrows; their molecular sizes are indicated.](image)
different cell types. The methylation status in fibroblasts (no expression), myelocytes (low level expression, no HS-3 site), and mature macrophages (high expression, HS-3 site present) was analyzed using the restriction enzyme isoschizomers HpaII (sensitive to cytosine methylation) and MspI (methylation-insensitive; 8). The specific recognition sequence for these enzymes (5'-CCGG-3') is present 5 times in the downstream region (Fig.2). One site is located within the MLDE region (see below) protected in footprint experiments (data not shown), which represents the only CpG site for eukaryotic methylation in the entire protected region. Equal restriction fragment patterns after MspI and HpaII digestion are indicative for demethylated DNA, whereas DNA methylation is revealed by the presence of higher molecular weight fragments after HpaII digestion. Southern blots of MspI or HpaII digested genomic DNA were probed with the BglII/HindIII fragment (Fig.2), which does not hybridize to the P lysozyme gene under stringent conditions. Digestion of genomic DNA from NIH3T3, RMB1, P388D1, and J774 cells either with MspI or HpaII revealed different methylation patterns for fibroblasts and immature cells than for mature macrophages (Fig.3). The restriction pattern of P388D1 and J774 DNA is the same for MspI and HpaII digestion (Fig.3), consistent with a lack of methylation in the entire region analyzed. Genomic digests of NIH3T3 and RMB1 DNA with MspI revealed a similar pattern, whereas HpaII digestion produced different patterns. Cleavage of NIH3T3 DNA revealed a single major component of about 800 bp (Fig.3). This fragment could be generated by cleavage at HpaII sites 2 and 6 (Fig.2), and would therefore indicate methylation of the CpG site within the footprint region. To prove this, NIH3T3 DNA was double-digested with HpaII and KpnI, which cuts asymmetrically within the proposed 787 bp fragment (Fig.2), resulting in a 600 bp fragment as expected. DNA from RMB1 cells is nearly undigestable with HpaII in the analyzed region (hybridization was detected only in a region corresponding to high molecular weight DNA; data not shown), indicating a strong DNA hypermethylation in this cell line. To ensure the DNA was accessible for HpaII digestion, RMB1 DNA was cleaved with HindIII prior to MspI or HpaII restriction digestion resulting in the observed 2.5 kb HindIII fragment which was not further digestible by HpaII (Fig.3). In addition, genomic DNA was mixed with plasmid DNA containing the pHS 2/4 fragment (see accompanying manuscript). In all samples tested complete HpaII-digestion of plasmid DNA was observed (data not shown).

![Fig. 4. Demethylation of the M lysozyme downstream region during in vitro differentiation of hematopoietic stem cells. 12 μg (30 μg for lane 5) of genomic DNA from FDCP-A4 cells after 0, 3, or 8 days of in vitro differentiation was digested with HindIII/MspI, or HindIII/HpaII and hybridized to the BglII/HindIII fragment (Fig.2). The fragments resulting from complete MspI or HindIII digestion are marked by arrows; their molecular sizes are indicated.](image)

![Fig. 5. Lack of transcriptional activation by the downstream enhancer after in vitro methylation. (A) Map of the M lysozyme gene downstream region showing the fragments used for the constructs tested in transient transfection assays: exon IV and Dnase I hypersensitive sites 1–6 (Möllers et al., accompanying manuscript) are boxed. CAT constructs are indicated below the map; cloning sites are: B=BgIII, H=HindIII, K=KpnI, and N=NheI. (B) CAT conversions of several CAT constructs relative to tkCAT activity (unmethylated) are shown after transfection of unmethylated or in vitro methylated (m) plasmid DNA in mouse L cells or P388D1 macrophages.](image)
These results clearly show that in the analyzed cell lines there is a correlation between high M lysozyme gene expression and the presence of the HS-3 site with demethylation of the CpG-sites in the MLDE footprint region and neighbouring sequences.

Demethylation of the downstream region during in vitro differentiation of hematopoietic stem cells

The establishment of HS sites (including the HS-3 site) was observed during in vitro differentiation of stem cells (Möllers et al., accompanying manuscript) and may reflect an increase in DNA accessibility to regulatory factors in the 3' flanking region of the M lysozyme gene. It is possible that changes in the methylation pattern coincide with the establishment of HS sites. To characterize the methylation pattern in the 3' flanking region of the M lysozyme gene, genomic DNA preparations were obtained at various times during in vitro differentiation of FDCP-A4 cells.

To study the kinetics of demethylation, genomic DNA was purified from the same batch of differentiated and undifferentiated cells used to determine the HS sites. The DNA from undifferentiated FDCP-A4 cells and from cells 3 or 8 days after stimulation of in vitro differentiation was digested with HindIII/MspI or HindIII/HpaII, electrophoresed, blotted, and hybridized to the BglIII/HindIII fragment. Thus, the presence of the 2500 bp HindIII fragment is indicative of methylated HpaII sites, whereas the presence of the 325, 282, 89/91 fragments and the absence of 2500 bp fragment is indicative of demethylation. The restriction patterns for MspI digestion are the same for all time points tested (Fig.4). While the DNA from undifferentiated cells or from cells 3 days after induction of differentiation is methylated at all HpaII sites; only the DNA from the most mature cells is completely demethylated (Fig.4). Even if double the amount of DNA was used, no smaller fragments were detectable in the HpaII digest of the DNA from cells 3 days after induction of differentiation (Fig.4, lane 5). Therefore, demethylation of a significant subpopulation of DNA molecules is excluded. Since the HS-3 site is already detectable at this time point (Möllers et al., accompanying manuscript), demethylation seems to occur simultaneously or somewhat later than the establishment of the HS-3 site.

Our results show that the 3' flanking region of the M lysozyme gene is fully methylated in pluripotent hematopoietic stem cells, and becomes demethylated during in vitro differentiation towards macrophages.

Enhancer function and protein binding are inhibited by CpG methylation

Since we found a clear correlation of gene expression with the methylation status of the endogeneous M lysozyme gene 3' flanking region, we investigated whether CpG methylation of the MLDE may influence its function. First we wanted to narrow down the enhancer element in transient transfection assays to investigate the importance of specific CpG sites. The first evidence for the presence of an important enhancer subelement in the M lysozyme 3' flanking region was the orientation dependent enhancement of CAT activity present in pH3tkCAT (this activity being higher in sense than in antisense orientation; Möllers et al., accompanying manuscript, and Fig. 5A, table 1) The data suggests that specific binding site(s) for activating proteins are located in the 3' half of the BglIII/HpaI fragment (+5.8/+6.2 kb) in pH3tkCAT . This was confirmed by transfecting NIH3T3 and P388D1 cells with the plasmids pH3.1tkCAT or pH3.2tkCAT, representing the 5' and 3' half of the BglIII/HpaI fragment contained in pH3tkCAT. Whereas the pH3.1tkCAT clone does not show any enhancer activity, the sequences contained in the pH3.2tkCAT clone are sufficient for strong enhancement of CAT activity (Table 1 and Fig. 5A, B). Since DNase I footprint experiments revealed only one prominent protected region in this fragment (see blow, Fig. 6) we inserted the double-stranded MLDE oligonucleotide (Fig. 5A), containing the footprint region, in front of tkCAT and transfected the resulting clones (pMLDEs and pMLDEs; containing the oligonucleotide in one or two copies in sense orientation) into mouse L cells and P388D1 macrophages. Table 1 shows that the single insertion, and to a greater extent, the double insertion of the MLDE oligonucleotide reveals an induction of the tk promoter in both cell lines. Therefore it was concluded that this 51 bp core enhancer is sufficient for transcriptional activation.

![Fig. 6. DNase I footprints on the MLDE sequence with nuclear extract of RMBI cells. Lanes without (-) or with extract (RMBI) are compared. The extent of the footprint is indicated next to the autoradiogram, including positions of hypersensitivity (shaded box): (A), sense strand; (B) antisense strand.](image)
To investigate the influence of DNA methylation on MLDE function, we transfected several constructs containing the core enhancer element with various amounts of flanking sequences (Fig. 5A), either methylated or unmethylated, into mouse L cells or P388D1 macrophages. Since the only CpG site located within the footprint region was present in an HpaII recognition site, we suspected that in vitro methylation using HpaII methylase should be sufficient to impair enhancer function. The plasmids ptkCATΔH/N and pSVtkCAT (Fig. 5A) were used as controls, because neither the tk promoter nor the SV40 enhancer are significantly inhibited by HpaII methylation (9). Fig. 5B shows that transfection of pHS2/4tkCAT, pHS3tkCAT, pHS3.2tkCAT, and pMLDEs tkCAT results in a 3 to 20 fold increase in CAT activity compared to ptkCATΔH/N in both cell lines, but when in vitro methylated DNA of the same constructs was used, no enhancer function was detected. The expression of ptkCATΔH/N and pSVtkCAT was not changed by methylation (Fig. 5B).

To analyze possible DNA-binding proteins on the MLDE-sequence we performed footprint assays. Different extracts from lysozyme expressing or non-expressing cells did not show any difference (data not shown), which agrees with the ubiquitous enhancer function in transfer experiments. As an example a footprint generated with RMB1 nuclear extract is shown (Fig. 6). The footprint extends over 50 bp, which defined the sequence used for generating the MLDE oligonucleotide (see above). Both strands exhibit a strong in vitro hypersensitive site. To test whether the loss of enhancement was due to inhibition of DNA binding of nuclear factors to the MLDE, we performed band shift assays using the 3P-labeled MLDE oligonucleotide, which was either unmethylated or in vitro methylated by HpaII methylase, and nuclear protein extracts from RMB1 or P388D1 cells. Fig. 7 shows two or possibly three complexes formed with the unmethylated oligonucleotide, which are only barely detected when the methylated oligonucleotide was used. Nuclear protein extracts from both cell types exhibited a similar binding pattern (Fig. 7, lanes 1, 2, 9, and 10). There is a high degree of specificity in the complexes formed with the unmethylated oligonucleotide, because they cannot be competed with oligomers containing binding sites for nuclear factors like SP1, AP1, AP2, OTF, or NFkB (Fig. 7C), despite the fact that all these binding sites possess some homology to sequences within the MLDE oligonucleotide. We compared the effect of methylation on protein binding affinity with competition experiments using increasing amounts of methylated or unmethylated MLDE oligonucleotide competitor. The methylated oligonucleotide competed 3 to 10 fold less efficiently than did the unmethylated oligonucleotide (Fig. 7A, lanes 3—8 and B, lanes 11—16, shown for both nuclear protein extracts). The importance of the CpG site for binding and specificity of complex formation is demonstrated with competition experiments using the mutated oligonucleotide MLDEmut., in which the CpG site is changed to two adenine residues. Compared to competition experiments using the unmethylated, wild type MLDE oligo, 10 fold higher amounts of the mutated form are necessary in order to compete both complexes (Fig. 7B, lanes 11—13 and 17—19). The faster migrating complex might represent binding of a nuclear factor to the CpG sequence, whereas the slower complex might contain additional factors requiring demethylated DNA and/or factors in the first complex to be bound.

These results clearly demonstrate that DNA methylation of a single CpG site in the MLDE core element, as found in non-expressing cells, is able to suppress protein binding and transcripational activation.

**DISCUSSION**

DNA methylation is involved in the tissue specific regulation of M lysozyme gene expression

The mouse M lysozyme gene is expressed only in mature cells of the granulocyte/macrophage lineage. The differentiation stage
specific expression of this gene is accompanied by well defined changes in a complicated pattern of DNaseI hypersensitive sites (Möllers et al., accompanying manuscript). Particularly remarkable in this context is the 3' flanking region of the gene: whereas the entire M lysozyme gene shows a very high degree of homology to the related P gene, which is expressed in different tissues, the 3' flanking region contains sequences specific for the M gene (8). In this region three HS sites appear during macrophage maturation, and one of these (HS-3) overlaps with the level of endogenous gene expression nor with the presence of the HS-3 site. The same lack of cell type specificity was found with nuclear factor binding to the corresponding sequences by in vitro footprinting experiments (Möllers, unpublished results). This suggests that some mechanism other than the simple presence/absence of specific transcription factors serves to regulate the tissue specific function of this enhancer element. DNA methylation has been shown to be one possible mechanism involved in the regulation of gene expression in developing cells (2, 3, 10; and references therein). Here we have investigated the possible role of DNA methylation for the tissue specific regulation of M lysozyme gene expression. Our results clearly demonstrate that methylation of a single CpG site within the MLDE inhibits proteins binding to the core element and its functional trans-activation. Methylation of this and neighbouring CpG sites was detected in fibroblasts, in hematopoietic stem cells, and even in myeloid immature cells; full demethylation being observed only in a late maturation stage. In other words, demethylation parallels or follows the establishment of DNaseI hypersensitivity and transcriptional activity. These results underscore the in vivo significance of a single CpG site, which when demethylated was demonstrated to inhibit the function of the MLDE. The inducibility of the endogenous M lysozyme gene in RMB1 myelocytes by demethylation emphasizes the important role of DNA methylation as a regulatory mechanism inhibiting gene expression in immature and unrelated cells. Demethylation might even control additional unknown regulatory elements of the lysozyme gene, since lysozyme expression in P388D1 cells can be further induced by demethylation, although the single CpG site within the MLDE core is already demethylated.

Similar experiments have been performed in other systems and in most cases a clear correlation between DNA methylation and the level of gene expression and/or enhancer function was demonstrated (recent examples include: 9, 11–15). One example should be emphasized because it also deals with developing hematopoietic cells. Seyferth et al. (16) reported that the inducibility of the Egr1 gene by antigen activation is inhibited in immature B cells by DNA methylation of the upstream region. Whereas demethylation, found in mature cells, leads to gene expression upon antigen activation. Inhibition of transcription by methylation events is not an invariable role as demonstrated by the fact that the ubiquitous transcription factor SP1 is inhibited neither in binding affinity nor transactivation when its recognition site contains methylated cytosines (17). This particular example may be due to a special role of methylation in the transcriptional regulation of housekeeping genes (12).

Many of the reports published to date concerning the effects of methylation on transcription have not combined in vivo and in vitro results. Studies on the functional influence of certain CpG sites on protein binding and transcriptional activation often do not satisfactorily demonstrate whether or not these CpG sites are indeed targets for methylation in vivo. On the other hand, many studies on methylation patterns in different cell types do not directly demonstrate the functional importance of this methylation. Our system is one of the few exceptions: since the only CpG site in the core enhancer element is located in an Hpall recognition site, we are able to correlate methylation patterns in vivo measured by Hpall digestion with functional impairment by in vitro methylation.

Recently, Wölfli et al. (18) reported that the DNA methylolation pattern of the promoter and 5'-untranslated region of the chicken lysozyme gene does not correlate with transcriptional activity. These authors studied the possible interference in the RNA polymerase complex interaction with DNA by CpG methylation. However, here we analyzed the inhibition of enhancer function by CpG methylation, a direct comparison of the results is difficult. In addition, it is possible that the chicken lysozyme gene, which is expressed in macrophages and oviduct, is regulated differently.

### Causal and/or temporal correlation between DNase I hypersensitivity, DNA methylation and transcriptional activation

One central question in studies on DNA methylation and gene expression concerns the causative connections between demethylation, the establishment of DNaseI hypersensitivity, and transcriptional activation. A possible indication of causal relationships may be derived from a correlation of the kinetics of changes in chromatin structure, demethylation, and the onset of transcription. This was demonstrated for the M lysozyme downstream enhancer during in vitro differentiation of hematopoietic stem cells towards macrophages, where complete demethylation of the CpG site in the MLDE core element clearly dates later than day 3. Since a slight intensification of the corresponding HS-3 site is already detectable at this time point (Möllers et al., accompanying manuscript), it could be inferred that the establishment of the HS-3 site may precede complete demethylation. This model is in good agreement to other publications: a clear correlation between the methylation state of the DNA and chromatin structure has been demonstrated in

### Table 2. Function and methylation of the mouse M lysozyme downstream enhancer; a comparison of in vivo and in vitro data.

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</table>

n.d. = not determined.
reports stating that DNA methylation may effect gene transcription by directly altering nucleosomal conformations (1, 19). Saluz et al. (20) showed that demethylation of a CpG site within an estrogen response element in the upstream region of the avian vitellogenin gene occurs in parallel to the appearance of the corresponding HS site. Transcriptional activation might be a consequence of demethylation, since MLDE function in transient transfections and in vitro nuclear factor binding are inhibited by methylation of the single CpG site within the MLDE. A direct inhibitory effect by methylation on transcription factor binding or repression of gene expression seems to be a common phenomenon (2), but may be overcome in special situations (11, 13). However, undermethylation by itself is not always sufficient to induce the formation of HS sites and to activate transcription, suggesting that in addition specific transactivating factors and/or flanking chromatin structures may be important (see above; 21). In this context, it is interesting to note, that it is not possible to induce the expression of the endogenous M lysosome gene solely by demethylation in fibroblasts, whereas in immature cells of the granulocyte/macrophage lineage a strong activation is achieved. This could be due to such additional factors which may not be present in different cell types.

Identification of a binding site for a new possibly ubiquitious transcription factor

Band shift assays with the MLDE core element revealed two complexes formed by nuclear protein extracts from myeloblasts and macrophages. Using a mutated form of this oligonucleotide (MLDE mut.) we identified a single CpG site to be absolutely necessary for nuclear factor binding. Methylation of the cytosine on both strands leads to a decreased binding affinity. This binding site is located at the edge of a palindromic structure extending over 24 bp (5'AGGAAAGTGAAGgtgggACTTCCg-3'). Whether this particular structure is important for protein binding and enhancer function remains to be clarified. Although there exist some homologies to other known binding sites for transcription factors like SP1 or NFkB, we demonstrated in competition experiments that the nuclear factor(s) binding to the MLDE oligonucleotide clearly differ from these. Thus, we identified a binding site for an unknown transcription factor. Because transcriptional enhancement resulting from the binding of this factor(s) to the MLDE core element is similar in every cell line tested (both in the mouse fibroblasts and macrophages described here, in HeLa cells and in HD11 chicken macrophages, unpublished results), we conclude that this factor(s) is either widespread or even ubiquitously expressed.

Summarizing, our results show a positive correlation of DNA methylation, DNase I hypersensitivity and transcriptional activation, giving a complete picture for a single CpG site during differentiation from pluripotent stem cells towards mature macrophages. Our system differs from other reports because it deals with a CpG site located downstream of the gene and since in vivo data correlate with in vitro protein binding and transient DNA transfection results.

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