Repression of the mouse M-lysozyme gene involves both hindrance of enhancer factor binding to the methylated enhancer and histone deacetylation

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ABSTRACT

In many cases, gene repression mediated by CpG methylation has been demonstrated. Two different mechanisms have been postulated to explain the repressive effect of methylated CpG DNA: establishment of a repressive chromatin configuration and inhibition of DNA binding of transactivating factors. Using the M-lysozyme gene, we analyzed gene expression, CpG of DNA binding of transactivating factors. Using the of a repressive chromatin configuration and inhibition repressive effect of methylated CpG DNA: establishment mechanisms have been postulated to explain the methylation has been demonstrated. Two different in many cases to correlate with transcriptional repression (1–5). Cytosine methylation of CpG dinucleotides has been shown in many cases to correlate with transcriptional repression (1–5). Housekeeping genes remain unmodified, whereas tissue-specific genes become methylated during embryogenesis after implantation. It has been shown that there is a correlation between tissue-specific demethylation and transcriptional induction of tissue-specific genes. In particular, during the granulocyte/macrophage differentiation, specific gene activation and regional DNA demethylation have been demonstrated (6–12). In the case of the myeloid-specific mouse M-lysozyme gene, we have provided functional evidence for the regulatory role of demethylation. The M-lysozyme gene is inactive in non-myeloid cells and in myeloid precursor cells, but is activated during granulocyte/macrophage differentiation. The downstream enhancer is methylated in inactive cell types and is demethylated during differentiation (8,13,14). Functionally, we have shown that in DNA transfection experiments, the methylated enhancer is inactive in transactivation, and that demethylation inhibits binding of the transcription factor GABP in vitro (8,15). Furthermore, the tissue-specific demethylation of the M-lysozyme enhancer is controlled by cis-acting sequences and is not caused by the transcription of an adjacent gene (15).

Two mechanisms have been suggested by which methylated DNA mediates transcriptional repression. First, several laboratories have demonstrated that a repressive conformation of chromatin is involved and that the strength of repression depends on the number of methylated CpGs (reviewed in 16). A molecular link between DNA methylation and chromatin inactivation was recently established (17,18). The MeCP2 protein, which binds specifically to methylated CpGs, has been found in a complex with histone deacetylases (17,18). Transfection assays with MeCP2 fusion proteins demonstrated repression of reporter genes which can be achieved with trichostatin A (TSA), a known inhibitor of histone deacetylases (17). Similar results were achieved after injecting effector as well as reporter plasmids into Xenopus oocytes (18).

Another mechanism of transcriptional repression by CpGs has been suggested by the finding that several transcription factors cannot bind to their specific DNA response elements when these elements are methylated (reviewed in 16). For the mouse M-lysozyme downstream enhancer, we have shown that a single CpG within the enhancer core (MLDE) is sufficient to regulate binding of heterotetrameric GABP (14). Even a single methyl group on the hemimethylated CpG is sufficient to interfere with GABP binding (15).

Therefore, we wanted to know which of the two repressive mechanisms is involved in silencing the lysozyme gene in inactive cells. To address this question, we have used myeloid cells that reflect different stages of differentiation and different lysozyme gene activities. Here we show that demethylation of the lysozyme gene results in an active in vivo enhancer complex and in gene induction, whereas inhibition of deacetylation neither demethylates the enhancer nor generates an enhancer protein complex. Nevertheless, gene activation is seen. These data suggest that both repressive mechanisms play a role in regulation of the M-lysozyme gene: chromatin deacetylation as well as the hindrance of transcription factors binding to DNA.

MATERIALS AND METHODS

Cell lines
RMB-3 and J774 1.6 (19) were grown in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) supplemented

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with 10% fetal bovine calf serum, 100 µg/ml streptomycin and 100 µg/ml penicillin. EL4 cells (ATCC TIB 39) were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine calf serum, 100 µg/ml streptomycin and 100 µg/ml penicillin.

Cells were treated with 5 µM 5-aza-2′-deoxycytidine (5-aza-dC) for 72 h or TSA (100–300 ng/ml) for the indicated times (for footprinting: 20–24 h).

RT–PCR

RNA was isolated using the Qiagen RNeasy Kit following the suppliers instructions. For reverse transcription, 0.5–1.5 µg RNA was isolated using the Qiagen RNeasy Kit following the RT–PCR protocol.

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RESULTS

Previously, we have characterized the mouse M-lysozyme downstream enhancer and found that the MLDE is bound by the heterotetrameric factor GABP (Fig. 1). Expression of the M-lysozyme gene is restricted to the granulocyte/macrophage lineage of hematopoietic cells (22), and the comparison of several cell lines revealed that in vivo factor binding to the GABP site is only seen in myeloid cells and correlates with the absence of methylation on the single CpG dinucleotide within the MLDE. All of the cell lines displaying a methylated CpG show no lysozyme expression and no in vivo footprint. In addition to the central core enhancer, flanking sequences contribute to enhancer strength, in particular the element upstream of the MLDE which is bound by the transcription factor NF-Y (Fig. 1). For the entire enhancer, the same correlation as for GABP has been found: no DNA methylation, in vivo factor binding and lysozyme gene activity (13–15, 23). From the total of five CpG dinucleotides within the full-length enhancer (15), only the single site within the MLDE element interferes with factor binding (15; O.Ammerpohl, unpublished results). Here we focus therefore on the methylation of this site, which can be analyzed by HpaII digestion.

Five different cell lines were analyzed for the effect of induced demethylation and the effect of inhibition of histone deacetylation on lysozyme gene activity, on MLDE methylation and on in vivo footprints. The cell lines chosen were Ltk− fibroblasts, inactive on lysozyme gene activity, on MLDE methylation and on in vivo footprint. All of the cell lines displaying a methylated CpG show no lysozyme expression and no in vivo footprint. In addition to the central core enhancer, flanking sequences contribute to enhancer strength, in particular the element upstream of the MLDE which is bound by the transcription factor NF-Y (Fig. 1). For the entire enhancer, the same correlation as for GABP has been found: no DNA methylation, in vivo factor binding and lysozyme gene activity (13–15, 23). From the total of five CpG dinucleotides within the full-length enhancer (15), only the single site within the MLDE element interferes in vitro with factor binding (15; O.Ammerpohl, unpublished results). Here we focus therefore on the methylation of this site, which can be analyzed by HpaII digestion.

Figure 1. Mouse M-lysozyme gene. The macrophage lysozyme gene locus is shown as a solid line; filled boxes indicate the exons 1–4 (roman numerals). The magnified region below contains the hypersensitive site HS3.2 (30). The footprint regions FP2 and FP3, which contain NF-Y and GABP binding sites, are indicated. The methylated cytidine within a HpaII site is marked by CH3. P1, P2 and P3 show the primers used for LM–PCR.

72°C, 22–26 cycles, radiolabeled P3 primer (GGAGCCTTC-TTTCTCTGGATCCCTTACATCCG) was added and a PCR was performed (1 min at 94°C, 1–2 min at 68°C, 3 min at 72°C, 4–6 cycles). The PCR products were precipitated and separated by sequencing gel electrophoresis.
examine whether the TSA effect is acting directly on the expression in all of the cell types is not changed (Fig. 2B). To lysozyme expression of mature macrophages and GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) was similarly analyzed as control, since this housekeeping gene is not induced by demethylation treatment (Fig. 2A). Expression of GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) was similarly analyzed as control, rather than a slight reduction seen for the other TSA inducible cell lines as well (not shown).

To test the methylation status of the critical CpG site within the GABP binding sequence after TSA or 5-aza-dC treatment, we tested all of the cell lines involved with methylated DNA.

72 h, the non-myeloid cells showed a very weak PCR band with lysozyme primers (Fig. 2A), whereas the myeloid precursor cells (RMB3) showed a very strong induction of lysozyme gene expression. The mature macrophage cell types being active in lysozyme expression cannot be induced further by 5-aza-dC (aza) for 72 h or with TSA for 10 h was isolated and used for RT–PCR as described above. Arrows indicate lysozyme- or GAPDH-specific bands.

Figure 3: 5-aza-dC but not TSA treatment leads to demethylation of genomic DNA in vivo. Different cell lines remaining untreated or treated with TSA (20 h) or 5-aza-dC (aza) (72 h). DNA was isolated and digested with KpnI only (KpnI) or double digested with KpnI and HpaII (HpaII) or KpnI and MspI (MspI). After LM–PCR, the products were separated on a sequencing gel. The large fragment (large) is specific for methylated and the smaller fragment (small) is indicative for unmethylated DNA.

lysozyme gene, we analyzed the kinetics of gene induction after TSA incubation (Fig. 2C). After only 3 h of TSA incubation, a maximal response on lysozyme expression is seen for the RMB3 precursor cells. This amount of lysozyme RNA is not changed even after incubating the cells for 24 h with TSA. Similar kinetics were seen for the other TSA inducible cell lines as well (not shown).

If chromatin deacetylation plays a role in methylated DNA-mediated gene repression, treatment of cells with a histone deacetylase inhibitor should induce lysozyme transcription in inactive cells, and should not change the lysozyme RNA level in mature macrophage cells. Indeed, after treatment with trichostatin-A (TSA), a potent inhibitor of histone deacetylases (24), lysozyme induction can be seen in LIk-, EL4 and RMB3 cells, whereas the lysozyme expression of mature macrophages and GAPDH expression in all of the cell types is not changed (Fig. 2B). To examine whether the TSA effect is acting directly on the lysozyme gene, we analyzed the kinetics of gene induction after TSA incubation (Fig. 2C). After only 3 h of TSA incubation, a maximal response on lysozyme expression is seen for the RMB3 precursor cells. This amount of lysozyme RNA is not changed even after incubating the cells for 24 h with TSA. Similar kinetics were seen for the other TSA inducible cell lines as well (not shown).

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Figure 4. 5-aza-dC but not TSA treatment leads to in vivo footprinting patterns specific for lysozyme expressing cells. Untreated EL4, RMB3, J774 and TSA or 5-aza-dC treated (aza) RMB3 cells were used for in vivo DNaseI footprinting as described in Materials and Methods. Black bars indicate protected regions. A hypersensitive site, specific for lysozyme-expressing cells, is marked by an arrowhead. (A) NF-Y binding region; (B) GABP binding region. In vivo footprints from cells treated with 20 µg DNaseI or 40 µg DNaseI are shown in lanes with odd or even numbers, respectively.

Therefore, we carried out in vivo footprint reactions over the NF-Y as well as the GABP response elements (Fig. 1). Focusing on the hematopoietic cell lines, the J774 mature macrophages serve as a positive control and show an obvious footprint over the NF-Y binding sequence (Fig. 4A). This indicates NF-Y binding, since identical in vivo and in vitro contact sites for NF-Y have been found (23,26). The negative control, EL4 T-cells, show no footprint over the same enhancer region. As expected, the untreated RMB3 precursor cells similarly show no footprint, whereas the 5-aza-dC-treated cells generate an in vivo footprint comparable to that of J774 cells. Interestingly, TSA treatment does not generate a NF-Y footprint, although in vitro enhancer methylation does not interfere with NF-Y binding (O.Ammerpohl, unpublished results). A similar result is seen for the in vivo footprint over the GABP binding sequence (Fig. 4B), which very likely reflects in vivo GABP binding, as judged from identical close contacts in vivo and in vitro (13,14). GABP binding is characterized by the DNaseI protection as well as a strong hypersensitive site as indicated by an arrowhead. Again, 5-aza-dC-treated RMB3 cells and J774 cells show the in vivo footprint. Thus, TSA-induced lysozyme expression in RMB3 cells is independent of the establishment of an active enhancer complex on the downstream enhancer, which had been shown to be the most prominent enhancing element (8).

**DISCUSSION**

The mouse M-lysozyme gene is a model system to study the effect of DNA methylation and demethylation. Several aspects contribute to the feasibility of this model system: the methylation of only a single CpG inhibits the binding of the core enhancer factor GABP and several stages of myeloid differentiation can be studied in different cell lines. Granulocyte/macrophage specific lysozyme gene activity correlates with enhancer demethylation and tissue-specific demethylation is controlled by cis-acting sequences (15). In addition to the important question of how tissue-specific demethylation is achieved, there is still a debate on the mechanism of gene repression mediated by methylated CpGs. The recent finding of the molecular connection between the methylated CpG binding protein MeCP2 and histone deacetylase complexes argues for an important role of histone deacetylation mediated by methylated DNA (17,18). These authors transfected or microinjected reporter genes repressed by recombinant MeCP2 derivatives. Repression was clearly relieved by TSA, thus showing the functional connection between MeCP2 and histone deacetylation. In general, histone acetylation and deacetylation play important roles in gene activation and inactivation (reviewed in 27).

In this study, we investigated whether such a functional connection can be seen for an endogenous gene in its natural differentiation-specific environment. In addition, such a test was important, since our previous results pointed to a different repressive mechanism: methylation mediated interference of GABP binding to the lysozyme enhancer (13–15). Therefore we focused our analysis on a single CpG within the MLDE for which we have shown that even a hemimethylation is sufficient to interfere with NF-Y binding (O.Ammerpohl, unpublished results). A similar result is seen for the in vivo footprint over the GABP binding sequence (Fig. 4B), which very likely reflects in vivo GABP binding, as judged from identical close contacts in vivo and in vitro (13,14). GABP binding is characterized by the DNaseI protection as well as a strong hypersensitive site as indicated by an arrowhead. Again, 5-aza-dC-treated RMB3 cells and J774 cells show the in vivo footprint. Thus, TSA-induced lysozyme expression in RMB3 cells is independent of the establishment of an active enhancer complex on the downstream enhancer, which had been shown to be the most prominent enhancing element (8).
Figure 5. Summary of lysozyme gene transcription, DNA methylation and presence of in vivo enhancer complexes. The results achieved with the different cell lines and TSA or 5-aza-dC treatment are shown. Methylation of the critical CpG interfering with GABP binding in the downstream enhancer is indicated (CH3). The heterotetrameric GABPβγδY complex (31) and the heterotrimeric NF-Y complex (32) are shown. The diagram is focused on the downstream enhancer, but the presence of other regulatory elements is possible.

enhancer factor loading after 24 h of TSA incubation. This shows that inhibition of deacetylation does not demethylate the lysozyme enhancer, which is in contrast to Neurospora, where a TSA-induced demethylation was observed (28). Furthermore, lysozyme gene activation is seen in the absence of an active enhancer complex on the downstream enhancer. Obviously the promoter or other regulatory elements may take over. Although we do not know on which part of the gene TSA is acting, we can clearly conclude that histone deacetylation is not solely involved in lysozyme silencing. Although enhancer methylation interferes with GABP binding (14,29), it does not interfere with NF-Y binding in vitro. Therefore, one could have envisaged a partial enhancer loading at least with NF-Y upon TSA treatment. This is not the case; in order to establish the enhancer complex, apparently the methyl groups have to be removed, allowing GABP to bind. This may subsequently change the enhancer chromatin or nucleosome positioning such that NF-Y and other factors can bind as well. This result indicates that DNA methylation-mediated repression of a single gene is achieved by both mechanisms: inhibition of enhancer establishment and inhibition by chromatin deacetylation.

The fact that even cell types usually never expressing lysozyme can be induced by inhibition of both maintenance methylase or histone deacetylases supports the idea of the evolutionary necessity of reducing transcriptional background noise (2). It has been proposed that large genomes require additional mechanisms that reduce transcriptional background activity. Such a background transcription in the absence of an established enhancer complex may be quite high as implied by the TSA-treated macrophage precursor cells.

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REFERENCES


NOTE ADDED IN PROOF

Using transfected DNA, Eden et al. have shown in a recent publication ([1998] Nature, 394, 842), that DNA demethylation and histone acetylation are functionally connected.