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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 demethylation and the *in vivo* formation of enhancer/protein complexes after inducing demethylation or inhibiting histone deacetylases. We show that transcription of a methylated and silent mouse M-lysozyme gene can be induced upon the inhibition of histone deacetylases in the absence of demethylation or *in vivo* transactivating factor binding to the enhancer. In contrast, DNA demethylation induces both gene activity as well as enhancer complex formation. Therefore, both mechanisms play a role in lysozyme gene repression mediated by methylated DNA: (i) the enhancer cannot be loaded with transacting factors; and (ii) histone deacetylation inhibits transcription.

INTRODUCTION

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 methylation 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 relieved 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, 6 pmol lysozyme specific primers Lys1/ Lys2 (Lys1: ATGAAG-ACTCTCCTGACTCTGGGAC; Lys2: CCACGGTTGTAGTT-TGTAGCTCGTG) or GAPDH specific primers GAPDH1/ GAPDH2 (GAPDH1: CGGAGTCAACGGATTTGGTCGTAT; GAPDH2: AGCCTTCTCCATGGTGGTGAAGAC), dNTPs (0.3 mM) and 3 U *Tth* polymerase (AGS) were incubated in 20 µl RT reaction buffer [67 mM Tris–HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween-20 and 1 mM $MnCl_2$] for 5 min at 85°C followed by 5 min at 60°C and 30 min at 72°C. For PCR amplification (30 s at 94°C, 45 s at 60°C, 80 s at 72°C, 25–29 cycles) 20 µl of polymerase buffer [335 mM Tris–HCl, pH 8.8, 83 mM $(NH_4)_2SO_4$, 3.75 mM EGTA, 25% Glycerin, 0.1% Tween-20], 250 pmol $MgCl_2$, 20 pmol dNTPs and 70 pmol of each primer (Lys1/Lys2 or GAPDH1/GAPDH2) were added and the volume was adjusted to 100 µl. PCR products were separated by agarose gel electrophoresis.

DNA isolation and digestion

$1–5 \times 10^7$ cells were washed twice in PBS, resuspended in 1 ml TE buffer and incubated with 1 ml phenol, pH 7.5–8.0 (Roth) for 15–20 min while gently mixing. After chloroform extraction, the DNA was precipitated and resolved in TE buffer or water. Digestion of DNA using Asp718 (Boehringer), *Hpa*II or *Msp*I (MBI fermentas) was performed as described previously (15).

DNase I *in vivo* footprint

DNaseI *in vivo* footprint was performed according to Rigaud *et al.* (20) with modifications described by Cappabianca *et al.* (21). Briefly, $\sim 2–3 \times 10^7$ cells were washed in PBS, resuspended in Ψ-Buffer (11 mM KPO_4, 108 mM KCl, 22 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mM ATP) containing 0.2% Igepal CA-630 (Sigma), divided into six portions and treated with different amounts of DNaseI (0–80 µg) for 3 min at 4°C. The DNA was isolated using phenol/chloroform extraction. About 1 µg DNA was used to perform LM–PCR.

LM–PCR

LM–PCR was performed as described before (13). Briefly, for first strand synthesis 1 µg digested DNA annealed to 1.0 pmol P1-Primer (TTTCGGCTGTGAGGCTCATAATTTACC) was incubated with Sequenase (Amersham) for 10 min. Ligation was performed adding 100 pmol L23 (GGTGACCCGGGAGATCT-GAATTC) primer annealed to L2 primer (GAATTCAGATC) and 2–3 U T4-ligase (MBI fermentas) for 15 h at 16°C. After PCR amplification with P2 (CCTTCAATGCTAGCGAGCTTCTT-TCTC) and L23 primers (1 min at 94°C, 1 min at 60°C, 2 min at 72°C, 22–26 cycles), radiolabeled P3 primer (CGAGCTTC-TTTCTCTGCATCCCTTCATCCGC) 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.

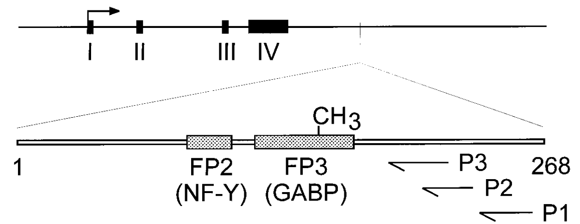

Figure 1. Mouse M-lysozyme gene. The macrophage lysozyme gene locus is shown as a solid line; filled boxes indicate the exons 1–4 (roman numerals). The magnified region below contains the hypersensitive site HS3.2 (30). The footprint regions FP2 and FP3, which contain NF-Y and GABP binding sites, are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

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* enhancer 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 *Hpa*II 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 for lysozyme expression, EL4 lymphocytic T-cells similarly lysozyme negative and RMB-3 myeloid precursor cells, which are characteristic for the differentiation stage before the onset of lysozyme transcription (19). Cell lines reflecting the mature macrophage stage were J774-1.6 and P388D1 (19). RT–PCR experiments were carried out with RNA from all of these cell lines and with lysozyme specific primers. As expected, Ltk^-, EL4 and RMB3 cells were negative for lysozyme expression, whereas P388D1 and J774-1.6 macrophage cells showed strong lysozyme gene activity (Fig. 2A). After treating the cells with 5-aza-dC for

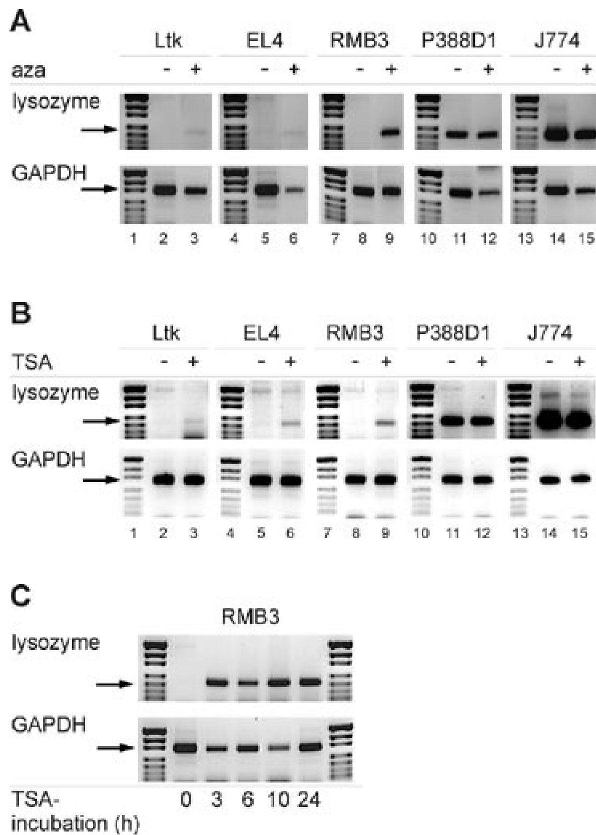

Figure 2. TSA and 5-aza-dC treatment activates lysozyme expression in non-expressing cells. (**A**) RNA from different cell lines untreated (–) or treated (+) with 5-aza-dC (aza) for 72 h or (**B**) with TSA for 10 h was isolated and used for RT–PCR as described in Materials and Methods. After agarose gel electrophoresis and ethidium bromide staining bands specific for lysozyme and GAPDH are marked by arrows. Lanes 1, 4, 7, 10 and 13 contain marker (MBI marker 23). (**C**) RNA from RMB3 cells treated with TSA for different incubation times was isolated and used for RT–PCR as described above. Arrows indicate lysozyme- or GAPDH-specific bands.

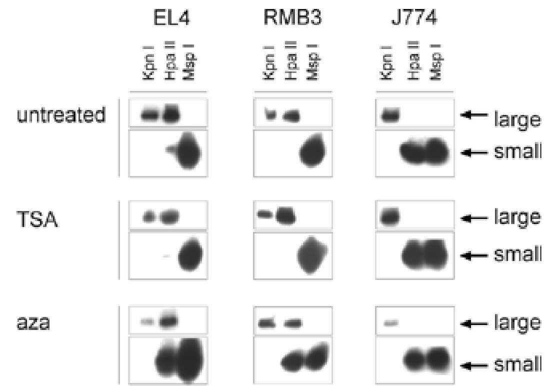

Figure 3. 5-aza-dC but not TSA treatment leads to demethylation of genomic DNA *in vivo*. Different cell lines remaining untreated or treated with TSA (20 h) or 5-aza-dC (aza) (72 h). DNA was isolated and digested with *Kpn*I only (*Kpn*I) or double digested with *Kpn*I and *Hpa*II (*Hpa*II) or *Kpn*I and *Msp*I (*Msp*I). 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.

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 activity. The mature macrophage cell types being active in lysozyme expression cannot be induced further by 5-aza-dC treatment (Fig. 2A). Expression of GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) was similarly analyzed as control, since this housekeeping gene is not induced by demethylation (O.Ammerpohl, unpublished results); rather a slight reduction could be seen (Fig. 2A).

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 Ltk^-, 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).

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 *Hpa*II digestion. *Hpa*II can only digest fully demethylated DNA. The results for the three important haematopoietic cell lines are shown (Fig. 3). The MLDE sequence within the T-lymphocytes (EL4) is resistant to *Hpa*II digestion, indicating the methylated state, whereas the control digestion with *Msp*I shows a complete digestion. The restriction enzyme *Msp*I recognizes the same DNA sequence as *Hpa*II, but is not sensitive to DNA methylation. This *Hpa*II resistance is maintained on TSA treatment, whereas the 5-aza-dC treatment results in an obvious demethylation. Complete demethylation is not seen, since this treatment does not actively remove methyl groups; rather the action of the maintenance methylase is inhibited during replication (25). Therefore, one has to expect that a small amount of MLDE sequences contain one originally methylated DNA strand and remain resistant to *Hpa*II digestion. A similar result is seen for the macrophage precursor cells RMB3: *Hpa*II resistance in the untreated cells as well as in the TSA treated cells, whereas the 5-aza-dC treatment results in a *Hpa*II digestion of the majority of MLDE sequences. This result clearly shows that the strong lysozyme induction by TSA treatment has no effect on methylation within the MLDE sequence. The mature macrophages (J774), which show unaltered amounts of lysozyme RNA after either treatment, are fully digestible with *Hpa*II in all conditions, confirming that the MLDE in J774 cells is demethylated (13) and that this demethylated state is not changed by either treatment.

Having shown that histone deacetylation plays a role in lysozyme gene repression, we wanted to know whether the inhibition of deacetylases allows the establishment of active enhancer complexes on the lysozyme downstream enhancer.

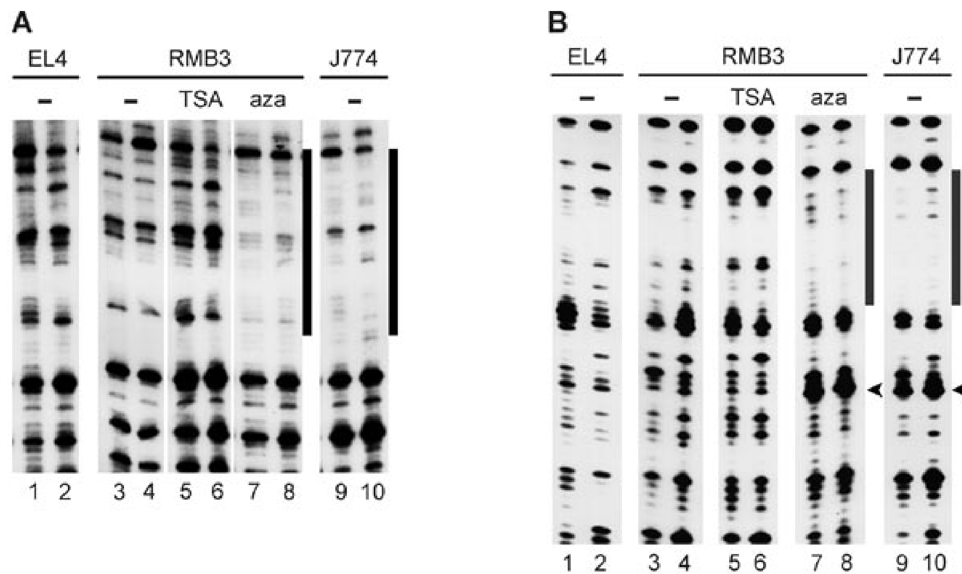

Figure 4. 5-aza-dC but not TSA treatment leads to *in vivo* footprinting patterns specific for lysozyme expressing cells. Untreated EL4, RMB3, J774 and TSA or 5-aza-dC treated (aza) RMB3 cells were used for *in vivo* DNaseI footprinting as described in Materials and Methods. Black bars indicate protected 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 GABP binding (15). Using the inhibitors of DNA methylation and of histone deacetylation, we could analyze whether gene activity, DNA methylation and the *in vivo* loading of the enhancer sequences with transcription factors would be affected. If DNA methylation mediates the biological effects via histone deacetylation only, the inhibition of either activity should allow the *in vivo* loading of the enhancer and induce gene activity. The results are summarized in Figure 5 and show clearly that the prevention of enhancer loading by the methylation of enhancer sequences can be separated from the repressive effects by deacetylation of histones:

TSA treatment of the myeloid precursor cells RMB3 induces the silent lysozyme gene in the absence of demethylation and of

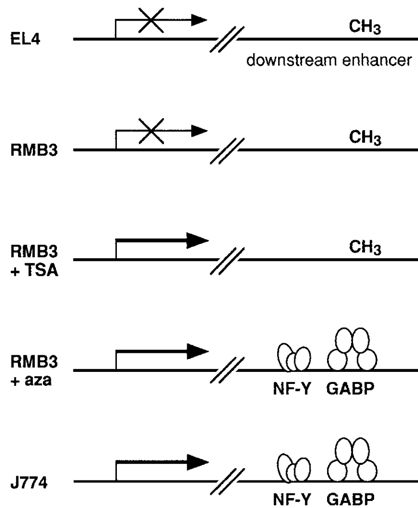

Figure 5. Summary of lysozyme gene transcription, DNA methylation and presence of *in vivo* enhancer complexes. The results achieved with the different cell lines and TSA or 5-aza-dC treatment are shown. Methylation of the critical CpG interfering with GABP binding in the downstream enhancer is indicated (CH_3). The heterotetrameric GABPα/β 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 gene 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 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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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.