Cis-elements Required for the Demethylation of the Mouse M-lysozyme Downstream Enhancer

Alexander Schmitz, Marc Short‡, Ole Ammerpohl, Christian Asbrandel, Joachim Nickelt, and Rainer Renkawitz

From the Genetisches Institut, Justus-Liebig-Universität, Heinrich-Buff-Ring 58-62, D35392 Giessen, Germany

The mouse lysozyme downstream enhancer was previously colocalized with the DNase I-hypersensitive site in the chromatin of mature macrophages. This hyper-sensitive site was shown to be macrophorad differentiation-dependent. Demethylation of CpG sequences within the enhancer is correlated with lysozyme expression in macrophages. Binding of the GABP heterotetrameric transcription factor to the enhancer core element (MLDE), only seen in vivo on the demethylated MLDE element in macrophages, is inhibited by DNA methylation. Here, we analyzed the DNA sequences required for demethylation. In electrophoretic mobility shift experiments we found that in addition to the complete methylated MLDE the hemimethylated form of the lower strand inhibits GABP binding as well. Therefore, GABP is unlikely to be the mediator of demethylation. In addition, we show by stable DNA transfections of methylated mouse lysozyme enhancer sequences that MLDE-flanking sequences are required for demethylation. We narrowed down these DNA elements to two short regions of 163 and 79 base pairs on either side of the MLDE, each of which is sufficient to mediate demethylation of the GABP site.

The mouse genome contains two lysozyme genes, a Paneth cell (P-lysozyme) and a macrophage-specific (M-lysozyme) gene generated by a gene duplication event (1). The M- and P-lysozyme genes are arranged in tandem with the coding regions separated by 5 kb (Fig. 1). Analysis of the M-lysozyme gene domain by DNase I digestion identified multiple hyper-sensitive (HS) sites in the 5′ and 3′ M-lysozyme gene-flanking regions in macrophage and myeloid precursor cell lines (Fig. 1) (2). Only a single site in the 3′-flanking region (HS3) is dependent on the differentiation state of the cell line and correlated with M-lysozyme gene expression (2). Transfection analysis of the flanking regions identified a single enhancer downstream of the M-gene which overlapped the HS3 site and is limited to the subregion HS3.2 (2, 3). Analysis of the HS3 region methylation state in M-lysozyme-expressing and non-expressing cells demonstrated a correlation between undermethylation of this region with both the presence of the HS3 site and expression of the M-lysozyme gene. Further fine mapping identified a central core enhancer (MLDE), which is bound by a heterotetrameric GABP complex (4). We found that GABP binding to the MLDE is methylation-sensitive (4). Thus, very likely, macrophage-specific demethylation of the single CpG dinucleotide within the MLDE is a mechanism to confer tissue-specific enhancer activity.

In other systems, methylation of CpG dinucleotides has been correlated with transcriptional inactivity as well (for review, see Refs. 5 and 6). In two cases, DNA transfections have identified quite complex DNA regions required for tissue-specific demethylation (7, 8). Recent achievements in demethylating DNA in vitro (9) showed an involvement of RNA and that tissue-specific proteins are required for the specificity of the reaction.

Here, we wanted to analyze the mechanisms mediating macrophage-specific demethylation of the single CpG site within the mouse lysozyme enhancer core MLDE. We identified two short DNA regions of 163 and 79 bp which are required and sufficient for demethylation. The two fragments are overlapping, but the sequence in common is not sufficient for demethylation.

EXPERIMENTAL PROCEDURES

Vectors—The pH3.6(1–1166)-tkCAT construct contains a 1.17-kb Asp718I/PstI fragment of the mouse lysozyme 3′-enhancer region including the MLDE sequence in a ptkCAT/HN vector (NdeI/HindIII fragment deleted from pBL-CAT2, (10)), plus additional restriction sites from the pks(−) polylinker of a subcloning vector. For pks(−)/HindIII construction, an EcoRV/PstI fragment from pMLg219 (4) was subcloned into the EcoRV/PstI site of pks(−). For pH3.2/tkCAT construction, an HphI/BamHI fragment of the pks(H3.2) plasmid was inserted into the SalI/BamHI site of ptkCAT/HN.

pMLDEtkCAT/HS3.2(121–171) (3) was reconstructed because of a sequencing error in the published oligonucleotide sequence. The corrected bases are shown in bold letters: 5′-CTATAGGTAAGCCAGGAAG-TAGAAGGTTGGGAGCTCCGGGAGAGAGTGGAACTCTGGGAGATAGTCAAG-3′ (153–188) construction, the synthetic double-stranded oligonucleotide (5′-GCCGGGAGAGAGTGGAACTCTGGGAGATAGTCAAG-3′) was inserted into the Klenow-filled SalI site of ptkCAT/HN. pH3.2(82–219) and pH3.2(146–219) were generated from pH3.2/tkCAT by exonuclease III deletions of the SpI/EcoRV-digested vector. HS3.2(1–181) and HS3.2(1–163) were constructed as follows. First, a modified pks(H3.2) (the SalI site 5′ to the insert was deleted by digesting with XhoI/XbaI, blunt ending with Klenow and religating) was digested with SalI plus PstI and treated with exonuclease III to generate deletions. After religation and verification of the deletions by DNA sequencing, the deleted fragments were subcloned into ptkCAT/HN using HindIII/XbaI.

Cell Lines—Mouse cell lines RAW264 (ATCC TIB71), M1 (ATCC
TIB192), RMB-3 and J774-1.6 (11) were grown in Dulbecco’s modified Eagle’s medium including 10% fetal bovine serum (12) and electroporated at 300 volts and 900 microfarads with an Easyject Gene Pulser (Eurogenetec). The cells were replated in a 15-cm tissue culture dish and selected for adherent cells. After 2–3 days, the pellet was resuspended 1 ml of phosphate-buffered saline, overlaid on 5 ml of Dulbecco’s modified Eagle’s medium, 0.75 ml medium (60% fresh medium, 40% RAW-conditioned medium). EL4 cells (ATCC TIB39) were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine calf serum, 100 µg/ml streptomycin, and 100 µg/ml penicillin.

In Vitro Methylation—Methylation was carried out in vitro on 40–50 µg of plasmid DNA by incubating with HpaII methylase (Fermentas 3 units/µg) overnight at 37 °C according to the supplier’s instructions. The reaction was terminated by incubating for 30 min at 65 °C, extracting with phenol:chloroform (1:1), and precipitating with EtOH. The degree of methylation was tested by HpaII digestion.

Stable Transfection, DNA Isolation and Digestion—For the RAW264 cell line, 105 cells were resuspended in 400 µl of Dulbecco’s modified Eagle’s medium including 10 µg of reporter plasmid, 1.5 µg of pPUR (12) and electroporated at 300 volts and 900 microfarads with an Easyject Gene Pulser (Eurogenetec). The cells were replated in a 15-cm tissue culture dish and selected for adherent cells. After 2–3 days, the cells were selected for puromycin resistance, using 6.5–7.5 µg puromycin per ml of medium (60% fresh medium, 40% RAW-conditioned medium). Growing colonies were expanded as cell pools for DNA isolation. For the EL4 cell line, 0.75 × 106 cells were resuspended in 400 µl of RPMI including 20 µg of reporter plasmid, 3 µg of pPUR, and electroporated at 250 volts, and 900 microfarads. The cells were transferred to a 15-cm dish. Selection for puromycin-resistant cells was started after 48 h using 7 µg of puromycin/ml of RPMI medium. After an additional 24 h a fetal calf serum centrifugation2 was performed as follows to remove the dead cells. The cells were centrifuged (800 rpm/5 min), and the cell pellet was resuspended in 10 ml of phosphate-buffered saline, separated into two vials, and centrifuged again (800 rpm/5 min). The cell pellet was resuspended 1 ml of phosphate-buffered saline, overlaid on 5 ml of fetal calf serum from the same batch that was used for the culture medium, and centrifuged again. The pellet with live cells was resuspended in 10 ml of RPMI including 7 µg/ml RPMI (60% fresh RPMI, 40% EL4 conditioned medium). The two vials were combined and transferred to a 15-cm cell culture dish. Growing colonies were expanded as cell pools for DNA extraction. The genomic DNA isolation was performed as described previously (13) with the following modifications. The DNA was precipitated with 1 volume of isopropl alcohol, removed with a flame sealed Pasteur pipette, rinsed in 70% EtOH, air dried, and dissolved in TE buffer (TE: 10 mM Tris-HCl, 1 mM EDTA, pH 7.6). About 30–50 µg of genomic DNA was digested twice with 8–10 units/µg of an appropriate restriction enzyme for 5 h and split into three aliquots, for Map1 digestion, HpaII digestion, and control, respectively. One µg from each of the three aliquots was used for LM-PCR.

Identification of Positive Clones and LM-PCR Assay—One µl of genomic DNA (0.5–2 µg of DNA) was PCR amplified with plasmid-specific primer pairs (ΔC-ΔT) with an annealing temperature of 64 °C or Forw2/25E (5 °C, 30 cycles), separated on an agarose gel, and visualized after staining. For tkCAT constructs or Forw19 for psKH3.2 were used on annealing temperatures of 64 °C and 50 °C, respectively. End labeling or psKH3.2 was performed at an annealing temperature as indicated with 32P end-labeled ΔC38 (5'-TGTTGGGGGTCGTTGGGGGCG-3') for tk constructs or L23/Forw.19 for psKH3.2 were used at annealing temperatures of 64 °C and 50 °C, respectively. End labeling or psKH3.2 was performed at an annealing temperature as indicated with 32P end-labeled ΔC38 (5'-TGTTGCTAATCAGCTGCAGTACAGGACAGCTTCG3'-70 °C) or S5 (5'-TGCCACCGCTCCGGCGCGCTTTT-3') (68 °C) corresponding to two plasmid constructs. The samples were electrophoresed on a 4–6% polyacrylamide, 8 M urea gel and autoradiographed. The size of the fragments was verified by control digests and 32P-labeled DNA marker.

Preparation of Nuclear Extracts, DNA Probes, Genomic Cytosine Sequencing, DNase I Footprinting, and Electrophoretic Mobility Shift Assay—Nuclear protein extracts from RAW264 and M1 cells were prepared as described by Nickel et al. (4). Genomic cytosine sequencing and DNase I footprinting were performed as described (13). For generation of hemimethylated MLDE probes, 100 pmol of oligonucleotide, synthesized with a single 5-methylcytosine CpG site, was annealed with the same amount of unmethylated complementary strand oligonucleotide. To generate methylated MLDE, complementary oligonucleotides were synthesized with 5-methylcytosine CpG sites and annealed. 30 pmol of the double-stranded oligonucleotides were labeled with γ-32P-ATP using T4 polynucleotide kinase according to the supplier's instructions (New England BioLabs). Labeled oligonucleotides were separated on a 5% polyacrylamide gel. After electrophoresis the gel was autoradiographed, and the band was excised. The DNA was eluted and used for electrophoretic mobility shift assay experiments, which were performed as described (4) with the modification that each sample contained 37 µg of yeast-RNA (Amersham).

RESULTS

A Single, Hemimethylated CpG Is Sufficient to Interfere with GABP Binding—Previously we have determined that methylation of the single CpG within the enhancer core MLDE is sufficient to regulate binding of heterotetrameric GABP (4). Therefore a possible mechanism for demethylation of this site might be that after DNA replication the GABP complex would be able to bind to the hemimethylated DNA. Thereby, the activity of the maintenance methylation could be inhibited, generating a fully demethylated DNA after a second round of replication. This type of competitive inhibition of the maintenance methylation has been suggested for the transcription factor Sp1 (15). Such a mechanism would require that the transcription factor GABP can bind at least to the hemimethylated DNA. Here, we tested this possibility by competing GABP binding with the double-stranded MLDE oligonucleotide with a single 5-methylcytosine CpG site in either the upper (sense) or lower (antisense) strand. Electrophoretic mobility shift assays were performed with nuclear extract from RAW macrophage cells as well as from M1 myeloblasts (Fig. 2). Both extracts generated several retarded complexes, with the upper complex (complex a, Fig. 2) consisting of the GABP heterotetramer bound to the palindromic DNA, whereas complex b is generated by an unknown protein (4). As expected, methylation of both strands of the single CpG site inhibits GABP binding and therefore is inactive in competition, whereas unmethylated DNA competes efficiently. Using the sense and antisense hemimethylated MLDE oligonucleotide as competitor, a strand-specific effect is seen. Lower strand (antisense) methylation inhibits GABP binding and is therefore ineffective in competition similar to the effect seen with the competitor methylated on both strands. In contrast, upper strand (sense) methylation shows almost no interference in GABP binding and is therefore an efficient competitor (Fig. 2).

Thus, an indirect demethylation by binding of GABP to the hemimethylated DNA after replication is unlikely since such a binding would only be possible for the hemimethylated upper strand. Such a mechanism might be conferred by another protein (complex b, Fig. 2). To test this possibility or to identify other
The mouse M-HS3.2 region containing CpG site 3 is bound. We have previously published that the central part of the light chain enhancer contains several CpG dinucleotides that are potential targets for methylation. It has been shown previously that one of these is located within the enhancer core MDLE and is specifically demethylated in macrophage cells (13). In contrast to macrophages, immature macrophages and T lymphocytes contain a 5-methylcytosine at this site on both strands. Here we extended this analysis on the entire HS3.2 region mediating full enhancer activity in DNA transfections (2, 3). Detection of methylated CpG dinucleotides was performed with cytosequencing in combination with LM-PCR (see “Experimental Procedures”). The genomic DNA was isolated from the following three mouse cell lines: mature macrophages (J774-1.6), immature macrophages (RMB-3), and T lymphocytes (EL4). Five CpG sequences are found within the HS3.2 region, sites 1, 2, 4, and 5 are shown in Fig. 3, and site 3 has been described previously (13). All of the sites follow the same methylation and demethylation pattern seen for site 3 (13); they are methylated in the T cell line and in the immature macrophages (Fig. 3). In contrast, the mature macrophage line J774-1.6 displays all of these sites in the cytosequencing reaction, indicating that all five sites are demethylated (Fig. 3).

We have previously published that the central part of the HS3.2 region containing CpG site 3 is bound in vitro by GABP. This in vitro footprint was identical for protein extracts from macrophage cells or from non-macrophage cells (13). Therefore, we analyzed the flanking sequences in an in vitro footprint reaction as well (Fig. 3C) and found additional sequences protected. Again, no major difference was seen for the EL4 and the J774 extract.

**Cis-elements for Demethylation**—To analyze the mechanisms involved in the macrophage-specific demethylation of the lysozyme downstream enhancer, we wanted to identify the DNA sequences required for the removal of methyl groups from the CpG dinucleotide within the enhancer core element. For these experiments we choose different DNA fragments from the downstream region harboring the chromatin-DNase I hypersensitive sites HS3–HS6 (see Fig. 1). This stretch of DNA sequence contains several CpG dinucleotides (see above), only one of which is part of the recognition sequence for the restriction enzyme HpaII (CCGG). This sequence is located in the center of the downstream enhancer within the binding site of the heterotetrameric transcription factor GABP (4). Therefore, this sequence could be easily methylated in vitro with the help of the HpaII DNA methylase. Such a methylated DNA was transfected into recipient cell lines, and stable transfectants were identified by their resistance to puromycin. Position effects on the methylation pattern of transgenes have been reported (16); therefore, we wanted to minimize the effects of particular integration sites by pooling about 50 colonies to analyze the methylation of the transfected DNA fragments. Such a strategy has been used successfully for the κ light chain enhancer (8). In a first series of experiments we tested a long (1.2 kb) fragment from the downstream region HS3/6 (see Fig. 1). We wanted to know whether such a DNA element after in vitro methylation could be found demethylated when integrated into the genome of a macrophage cell line. After transfection into RAW cells and subsequent isolation of the DNA from pooled cell clones, the genomic DNA was digested either with the restriction enzyme HpaII or with the restriction enzyme MspI. MspI digestion serves as a control, since the enzyme cuts both methylated DNA and unmethylated DNA. In contrast, digestion with HpaII is only possible with unmethylated DNA. To avoid difficulties in restriction enzyme digestion caused by the viscosity of the large genomic DNA, we routinely used an additional restriction enzyme to digest the DNA into larger fragments (NdeI, BamHI, PstI; see restriction site map in Fig. 4). In addition, the amount of the large fragment generated by the first restriction enzyme represents the undigested (i.e. methylated) material after HpaII digestion. Thus, the efficiency of demethylation can be judged from the ratio of intensities of the “smaller” fragment (HpaII-digested) relative to the “larger” fragment (HpaII-resistant). In contrast, the relative intensities of bands generated in different digestions (different lanes) are of no relevance. The fragments were visualized by LM-PCR with primers specific for the transfected DNA. Cell pools generated with the methylated HS3/6 fragment always showed an almost complete digestion with the enzyme HpaII (Fig. 4), indicating a successful demethylation of the DNA. At this point we were worried about a possible artifact, since Weiss et al. (9) showed that nonspecific demethylation activity is present during the genomic DNA isolation procedure even after proteinase K digestion. On the other hand, these authors showed that EDTA abolished this activity completely. We tested this possibility and found that our extraction procedures in the presence of 5 mM EDTA did not allow additional nonspecific in vitro demethylation (data not shown). In addition, as seen below, depending on the DNA sequence tested, we found the methylated CpG being preserved after transfection and DNA isolation.

Next we focused just on the 200-bp enhancer fragment HS3.2 and found that this short fragment was similarly demethylated (Fig. 4). This was surprising since a similar analysis of the demethylation within the intronic κ chain enhancer showed that more than 1 kb of DNA was required for successful demethylation in lymphocytes (8). Similarly, in transient transfections of the methylated α-actin promoter about 800 bp of DNA was required to demonstrate demethylation in myelocytes (7). We wondered whether the transcriptional activity of the transfected reporter gene may cause or influence the demethylation or whether the observed demethylation on the short HS3.2 fragment would be seen in the context of a different vector as well, which does not show transcriptional activity of its own in eukaryotic cells. Therefore we introduced the HS3.2 fragment into the prokaryotic Bluescript vector pSK. The
demethylation analysis of the pooled transfection clones showed a demethylation similar to that observed in conjunction with the tkCAT reporter (Fig. 4). Therefore, we were assured that the demethylation observed is not the consequence of transcriptional activity in its vicinity, but rather that demethylation is caused by the HS3.2 fragment itself.

Previously we have shown (13) that demethylation of the endogenous downstream enhancer is restricted to macrophage cells. T lymphocytes that do not express the lysozyme gene show a methylation of the CpG site within the downstream enhancer HpaII sequence. Therefore, we wanted to know whether the transfected methylated DNA shows this preference for demethylation in macrophages as well. We transfected methylated HS3.2 DNA as well as the methylated GABP-binding fragment (MLDE) into a T cell line (EL4). Analysis of the transgene showed that the majority of HS3.2 fragments was

![Fig. 3. All of the CpG dinucleotides within the mouse M-lysozyme downstream enhancer are demethylated in macrophage cells. Panel A, nucleotide sequence of the mouse M-lysozyme HS3.2 region. All of the CpG sequences are numbered consecutively. Panel B, DNA isolated from RMB-3, EL4, and J774-1.6 cells was used for in vivo cytosine sequencing, which only detects unmethylated cytosines in addition to thymines. The different cell types are indicated, and CpG dinucleotides demethylated in J774-1.6 cells are numbered corresponding to panel A. The single HpaII site (site 3) is not shown but has been characterized previously (13). Panel C, in vitro DNase I footprint in the presence of extracts from the indicated cell lines or without extract (−). Protected regions are marked.](#)
resistant to HpaII digestion and therefore has maintained the methylation at this site (Fig. 4C). Similarly, propagation of the methyl group was seen for the transfected MLDE sequence as

FIG. 4. The mouse M-lysozyme downstream enhancer region is sufficient in mediating macrophage-specific demethylation. Panel A, map of the HS3/6 and the HS3.2 regions (boxed) within the vector sequences (thin line). The MLDE is indicated by dark shading. Relevant restriction enzyme sites are shown, and the plasmid-specific primers for first strand synthesis are marked (arrows). Panel B, plasmids containing the HS3/6 enhancer region or the HS3.2 fragment (within the tkCAT or psk vectors) were methylated in vitro using HpaII methylase and were cotransfected with a plasmid coding for puromycin resistance into RAW264 macrophage cells. Genomic DNA of stable clone pools was digested with the indicated restriction enzymes. Fragments were visualized using LM-PCR (see “Experimental Procedures”). End labeling was performed with 32P-labeled primer S5, and samples were electrophoresed on a 6% polyacrylamide gel and autoradiographed. The relative intensities of the smaller fragment versus the larger fragment in the HpaII lanes indicate that almost all of the molecules are HpaII-digested and therefore demethylated. Panel C, fragments HS3.2 and MLDE were methylated in vitro and transfected into EL4 T cells. Analysis was performed as described in panel B and revealed that the majority of the molecules remains methylated since they are undigestible by HpaII.

well (Fig. 4C). This tissue specificity of the HS3.2 fragment encouraged us to use even shorter enhancer fragments to delineate the DNA elements sufficient for demethylation in macrophages. From the HS3.2 region we generated four overlapping fragments that were methylated by HpaII methylase and transfected into RAW cells similar to the previous experiments. Surprisingly, all of these subfragments showed a demethylation for the majority of the molecules (Fig. 5). Even the shortest fragment (HS3.2(146–224)), only 78 bp in length, was HpaII-digestible. The other extreme, fragment HS3.2(1–163), showing only an overlap with the previous fragment of 17 bp, was digestible by HpaII as well. Such a result may be explained by two different mechanisms. Either the short region just overlapping between the two fragments HS3.2(146–224) and HS3.2(1–163) would contain all of the information required to direct the demethylation activity to this CpG dinucleotide, or redundant cis-elements for demethylation occur in the enhancer, one in HS3.2(146–224) and one in HS3.2(1–163). To distinguish between these two possibilities we transfected the MLDE (4), which contains a little more than the overlapping sequence (bp 120–171), into RAW cells (Fig. 6). The transgene fragment was not digestible at all with HpaII, in contrast to the complete digestion with the control enzyme (MspI) and in contrast to the unmethylated transfection analyzed by HpaII digestion (Fig. 6). Similarly, we tested the demethylation of the fragment HS3.2(153–188). In addition to the demethylation site, this fragment contains a footprint region seen in vivo in macro-
phage cells (footprint 4). Methylation of this fragment shows a result similar to that seen with the MLDE fragment; the methyl group is maintained in the transgene fragment (Fig. 6).

Other attempts to identify the nucleotide sequences required for demethylation more precisely resulted in variable degrees of demethylation. Thus we can conclude that both of the fragments HS3.2(146–224) and HS3.2(1–163) contain all of the cis-elements required for demethylation (Fig. 7) and that these elements very likely are redundant within the HS3/6 downstream enhancer region of the mouse M-lysozyme gene.

DISCUSSION

Many examples have been identified linking sequence-specific DNA demethylation with differentiation of a particular tissue or cell type (for review, see Refs. 5 and 6). In these cases, it has been shown that the differentiation-dependent expression of a tissue-specific gene is correlated with the demethylation of flanking sequences. Such a demethylation is usually restricted to a specific region or at least to specific genes. For example, expression-linked demethylation has been seen for the genes coding for chicken vitellogenin, human dihydrofolate reductase, mouse collagen IV, rat α-actin, mouse κ chain, human estrogen receptor, human galectin-1, or mouse pyruvate dehydrogenase E1α subunit, to name only a few (7, 8, 17–22).

Other genes, not expressed in this particular tissue, remain methylated. Such a correlation has been found for the mouse M-lysozyme gene as well. The M-lysozyme gene is inactive in non-macrophage cells and shows a methylated CpG dinucleotide within the single HpaII recognition sequence of the downstream enhancer (3). It has been shown that during the differentiation of the multipotent FTCP-A4 cell line toward macrophages the enhancer loses its methyl groups (3). Similarly, the human lysozyme gene has been found to be demethylated depending on the differentiation as seen in ex vivo cultures of hematopoietic progenitor cells, whereas the gene coding for myeloperoxidase showed unaltered demethylation (23). Other myeloid-specific demethylation events have been shown as well to correlate with transcriptional activity, such as the c-fms gene and the regulatory region of the tumor necrosis factor-α gene (24, 25).

Many of the published examples could not distinguish between a role for the demethylation being required for transcriptional activity or a possible demethylation-inducing function mediated by transcription. In some cases, methylation-dependent repressor binding or inhibition of transcription factor binding could be demonstrated (5, 26–29). For the mouse lysozyme gene, it was shown that within the core part of the downstream enhancer the heterotetrameric transcription factor GABP is required for full enhancer activity (4). In addition, it was demonstrated that the methylation of a single CpG dinucleotide within the enhancer core region inhibits in vitro DNA binding of GABP (4). A similar sensitivity in methylated DNA binding was demonstrated for GABP and other ETS proteins in the context of binding sites that differ from the mouse lysozyme GABP binding site (30–32). This suggests that demethylation of this enhancer is a prerequisite for enhancer activity and therefore, for transcription. Here we show that the cis-element-dependent demethylation can be observed independent of the type of the neighboring DNA or promoter context. The 224-bp fragment HS3.2 can be stably transfected either fused to a eukaryotic reporter gene or to a prokaryotic vector and will be demethylated in both cases. Therefore, the lysozyme downstream enhancer confers at least two functions. One function is

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3 M. Short, unpublished data.
to mediate the demethylation during differentiation, and another function is to activate gene transcription by the bound enhancer factors. A similar dual activity has been demonstrated for the α-actin promoter and the κ enhancer (7, 8).

Our transfection data suggest that the MLDE fragment within the downstream enhancer is not sufficient to mediate demethylation. This fragment harbors the single HpaII site and binds the heterotetrameric GABP factor in the absence of methylation. Here we have shown that even a single methyl group on the lower strand is sufficient to inhibit GABP binding. Since GABP binding is impaired even by hemi-methylated DNA, this factor cannot be the cause for demethylation. This is in contrast to the mechanism observed in the context of Sp1 binding (15). Sp1 is able to bind methylated DNA and after replication prevents the maintenance methylase from modifying the newly synthesized DNA strand. Such a Sp1-like activity could have been envisioned to be utilized by an unknown factor that has been found to bind to the GABP response element even in the case of a fully methylated DNA (4). If this factor would indeed play such a role, this function would not be sufficient for demethylation, since we have shown that the methylated MLDE fragment is not demethylated (Fig. 6).

Within the group of fragments mediating demethylation, there seems to be a bias in demethylation efficiency: all of the fragments extending up to the position 224 (Fig. 7; i.e. fragments HS3/6, HS3.2, 82–224, 146–224) mediate 90–100% demethylation. In contrast, fragments with a downstream deletion (Fig. 7; i.e. fragments 1–181, 1–163) mediate demethylation for only 70–80% of the molecules. Nevertheless, two different sets of fragments, overlapping in the GABP site only, confer demethylation. Computer analysis of the sequences flanking the GABP site did not reveal any consensus in common, which otherwise might have been an indication for trans-acting proteins required for demethylation. In addition, in vitro footprinting showed only one protected region in addition to the GABP site (Fig. 3C).

One of the minimal regions required and sufficient for demethylation is only 78 bp in length (HS3.2(146–224)) (Fig. 7). None of the sequences analyzed for conferring demethylation in the other model systems could be delineated to such a small fragment. For both the α-actin promoter and the κ enhancer, DNA fragments of 800 bp to more than 1000 bp in length were required for demethylation (7, 8). Despite the complexity of the κ enhancer it could be shown that the absence of a single enhancer factor (nuclear factor-κ) is sufficient for demethylation, since we have shown that the methylated MLDE fragment is not demethylated (Fig. 6).

Taken together, these and our results demonstrate that the demethylation activity is mediated via enhancer elements and may be organized in a manner similar to that of the enhancer elements; that is, specific modules within the enhancer regions are either sufficient by themselves to mediate demethylation or have to act in combination with other modules.

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REFERENCES

1. Cross, M., and Renkawitz, R. (1990) EMBO J. 9, 1283–1288
2. Möllers, B., Klages, S., Wedel, A., Cross, M., Spooner, E., Dexter, T. M., and Renkawitz, R. (1992) Nucleic Acids Res. 20, 1917–1924
3. Klages, S., Möllers, B., and Renkawitz, R. (1992) Nucleic Acids Res. 20, 1925–1932
4. Nickel, J., Short, M. L., Schmitz, A., Eggert, M., and Renkawitz, R. (1995) Nucleic Acids Res. 23, 4785–4792
5. Tate, P. H., and Bird, A. P. (1993) Curr. Opin. Genet. Dev. 3, 226–231
6. Eden, S., and Cedar, H. (1994) Curr. Opin. Genet. Dev. 4, 255–259
7. Paroush, Z., Keshet, I., Yisraeli, J., and Cedar, H. (1990) Cell 63, 1229–1237
8. Lichtentausch, M., Keini, G., Cedar, H., and Bergman, Y. (1994) Cell 76, 913–923
9. Weiss, A., Keshet, I., Razin, A., and Cedar, H. (1996) Cell 86, 709–718
10. Baniahmad, A., Steinier, C., Kohne, A. C., and Renkawitz, R. (1996) Cell 61, 505–514
11. Leenen, P. J. M., Jansen, M. A. C., and Van Ewijk, W. (1986) Differentiation 32, 157–164
12. de la Luna, S., Soria, L., Pulido, D., Ortín, J., and Jimenez, A. (1988) Gene (Amst.) 67, 121–126
13. Short, M. L., Nickel, J., Schmitz, A., and Renkawitz, R. (1996) Cell Growth & Differ. 7, 1545–1550
14. Mueller, R. P., and Wold, B. (1989) Science 246, 780–786
15. MacLeod, D., Charlton, J., Mullins, J., and Bird, A. P. (1994) Genes Dev. 8, 2282–2292
16. Korti, P. A., Mangel, L., Schmitz, B., and Doerrler, W. (1996) Transgenic Res. 5, 235–244
17. Philipson, J. N., Gruber, M., and Ab, G. (1985) Biochim. Biophys. Acta 826, 186–194
18. Shimada, T., Inokuchi, K., and Nienkuis, A. W. (1987) Mol. Cell. Biol. 7, 2830–2837
19. Burbele, P. D., Horikoshi, S., and Yamada, Y. (1990) J. Biol. Chem. 265, 4839–4843
20. Fergusson, A. T., Lapidus, R. G., Baylin, S. B., and Davidson, N. E. (1995) Cancer Res. 55, 2279–2283
21. Benvenuto, G., Carpinteri, M. L., Salvatore, P., Cindolo, L., Bruni, C. B., and Chiaramoni, L. (1996) Mol. Cell. Biol. 16, 2736–2743
22. Iannello, R. C., Young, J., Sumarsono, S., Tymms, M. J., Dahl, H. M., Gould, J., Hedges, M., and Kola, I. (1997) Mol. Cell. Biol. 17, 612–619
23. Lubbert, M., Brugger, W., Mertelsmann, R., and Kanz, L. (1986) Blood 67, 447–455
24. Felgner, J., Kriege, H., Heidorn, K., Jäger, K., Heusink, E., Zechen, F., Radeau, H. J., and Parwaresch, M. R. (1990) Leukemia 4, 420–425
25. Takei, S., Fernandez, D., Redford, A., and Toyoda, H. (1996) Biochem. Biophys. Res. Commun. 220, 606–612
26. Boes, J., and Bird, A. (1991) Cell 64, 1123–1134
27. Jost, J. P., Saluz, H. P., and Pawlak, A. (1991) Nucleic Acids Res. 19, 5771–5775
28. Saluz, H. P., Peavers, I. M., Jirney, J., and Jost, J. P. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 6697–6700
29. Robertson, K. D., Hayward, S. D., Ling, P. D., Samid, D., and Ambinder, R. F. (1995) Mol. Cell. Biol. 15, 6150–6159
30. Yokomori, N., Kobayashi, R., Moore, R., Sueyoshi, T., and Negishi, M. (1995) Mol. Cell. Biol. 15, 5355–5362
31. Gaston, K., and Fried, M. (1995) Gene (Amst.) 157, 257–259
32. Desnet, C., Debaker, O., Faros, I., Larpag, C., Brasseur, F., and Boon, T. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 7149–7153
33. Kirillov, A., Kistler, B., Mostoslavsky, R., Cedar, H., Wirth, T., and Bergman, Y. (1996) Nat. Genet. 13, 435–441
34. Jost, J. P. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 4684–4688
35. Jost, J. P., Siggmann, M., Sun, L., and Leung, R. (1995) J. Biol. Chem. 270, 9754–9759
36. Sper, M., Theberge, J., and Bozovic, V. (1995) J. Biol. Chem. 270, 12860–12869
37. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K. (1988) Current Protocols in Molecular Biology, John Wiley & Sons, New York
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