Biol. Chem. Hoppe-Seyler, Vol. 377, pp. 47–56, January 1996 · Copyright © by Walter de Gruyter & Co · Berlin · New York

Differential Sensitivity of Zinc Finger Transcription Factors MTF-1, Sp1 and Krox-20 to CpG Methylation of Their Binding Sites

Freddy Radtke, Martin Hug, Oleg Georgiev, Koichi Matsuo and Walter Schaffner*

Institut für Molekularbiologie II der Universität Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland

* Corresponding author

Cytosine methylation at CpG sites is often negatively correlated with mammalian gene activity. Many transcription factors whose DNA binding site contains one or more CpG dinucleotides are no longer able to efficiently bind DNA when the site is methylated. A notable exception is the zinc finger factor Sp1 which binds DNA and activates transcription even when its binding site is methylated. Here we show that two other zinc finger factors, MTF-1 and Krox-20, can also bind to CpG methylated sites. MTF-1 regulates metallothionein gene transcription by binding to a number of metal responsive elements (MREs), and Krox-20 regulates Hox genes during hindbrain segmentation. However, a refined analysis of MTF-1/MRE binding shows that methylation is not tolerated at every binding site: the highest affinity site in the mouse metallothionein I gene, MREd, is unaffected by methylation, while two other MRE sites with CpGs at different positions are rendered partially or completely nonfunctional by methylation. Both methylation sensitive and insensitive factors/binding sites are likely to determine the developmental expression pattern of a gene.

Key words: DNA methylation / Krox-20 / Metal-responsive element (MRE) / MTF-1 / Zinc finger transcription factors.

Introduction

Many binding sites of transcription factors contain CpG dinucleotides. The target of modification by vertebrate DNA methyltransferase is cytosine in CpG dinucleotides (Bestor and Ingram, 1983; Simon *et al.*, 1983; reviewed by Hergersberg, 1991; Leonhardt and Bestor, 1993), and DNA methylation can affect or regulate binding of transcription factors to CpG-containing sites. Transcription factors whose binding site contains at least one CpG dinucleotide have been tentatively classified into two functional groups, methylation sensitive or insensitive, based on the effect of CpG methylation on their DNA binding affinity (Ehrlich and Ehrlich, 1993). Methylation sensitive factors include CREB and E2F, which bind to their respective sites, TGACGTCA (Iguchi-Ariga and Schaffner, 1989) and TTTCGCG (Kovesdi *et al.*, 1987), only when these sites are unmethylated. To our knowledge only one methylation insensitive transcription factor, Sp1, has been described so far. Sp1 is a ubiquitously expressed protein that drives a wide range of vertebrate promoters, notably of housekeeping genes (Kadonaga *et al.*, 1987; Courey and Tjian, 1992). We and others have observed that Sp1 can bind to its CpG containing sites even when they are methylated (Höller *et al.*, 1988; Harrington *et al.*, 1988; Ben-Hattar *et al.*, 1989). In addition, we could show that transcription efficiency both *in vitro* and *in vivo* was not impaired by methylation of the Sp1 binding site (Höller *et al.*, 1988). In γ-globin genes, Sp1-like transcription factors bind more tightly to CpG methylated sites and may thus contribute to repression by competing for the binding of a specific activator (Jane *et al.*, 1993; Sengupta *et al.*, 1994; see also Joel *et al.*, 1993). In a straightforward scenario where promoter activity is dependent on methylation sensitive transcription factors, transcription is completely blocked when the gene is methylated (reviewed by Hergersberg, 1991). Transcriptional activity can, at least temporarily, remain unaffected by methylation if a promoter is driven either by methylation insensitive factors, or by transcription factors whose binding sites lack a CpG dinucleotide. However, methylation outside of transcription factor binding sites can still indirectly inhibit binding of the factor to target sites, as a result of steric hindrance by methyl CpG binding proteins and/or tightly packed chromatin structures (Tazi and Bird, 1990; Boyes and Bird, 1991; reviewed by Hergersberg, 1991).

MTF-1 is a transcription factor implicated in activating mammalian metallothionein genes. We have reported elsewhere (Heuchel *et al.*, 1994) that in mouse embryonic stem cells that lack MTF-1 by targeted gene disruption neither basal nor heavy metal induced transcription of the metallothionein I and II genes is detectable. The cDNA sequences encoding both mouse and human MTF-1 (Radtke *et al.*, 1993; Brugnera *et al.*, 1994) revealed a DNA binding domain composed of six zinc fingers of the TFIIIA- or Cys_2-His_2 type (Kaptein, 1991). This type of finger is found also in other transcription factors such as Sp1 (Kadonaga *et al.*, 1987), Krox-20 (Chavrier *et al.*, 1988) and Krox-24/Zif268 (Lemaire *et al.*, 1988; Christy *et al.*, 1988). Mouse Krox-20 was originally identified as an immediate-early gene whose expression is induced following serum stimulation (Chavrier *et al.*, 1988) and was subsequently shown to regulate HoxB2 expression during rhombomere formation in mouse embryogenesis (Sham *et al.*, 1993).

Krox-20 has an identical DNA binding domain to Krox-24/Zif268 (Lemaire *et al.*, 1988). We wondered whether not only Sp1 but also other factors with Cys_2-His_2 zinc fingers were insensitive towards cytosine methylation in the binding site. Here we demonstrate that three zinc finger proteins, MTF-1, Sp1 and Krox-20, can bind to their high affinity CpG-containing binding sites irrespective of methylation. However, a closer inspection showed that the binding of MTF-1 was affected when other, lower affinity MRE sites with CpGs at different positions were used, thus indicating that methylation insensitivity is not a general property of Cys_2-His_2 zinc finger factors.

Results

Three Zinc Finger Proteins Can Bind to Their Recognition Site Irrespective of Methylation

Sp1 has three zinc fingers of Cys_2-His_2 type and recognizes the so-called GC boxes, GGGGCGGGGC. The consensus sequence derived from a number of Sp1 binding sites (Briggs *et al.*, 1986; Ben-Hattar *et al.*, 1989) is 5′-**G**/T-**G**/A**GGC**/A**GG**/T**G**/A**G**/A**C**/T-3′. It has previously been shown by us and others that Sp1 binds to its recognition sites even when the central CpG dinucleotide is methylated. Sp1 binding sites analyzed in these experiments were synthetic 5′-CTTCGGGCGGAGTTAC-3′ (Höller *et al.*, 1988), 5′-GCCGGGGCGGGGCTTC-3′ from the human metallothionein IIA promoter (Harrington *et al.*, 1988) and 5′-CGCTGGGCGGGGCCGG-3′ from the herpes simplex virus thymidine kinase promoter (Ben-Hattar *et al.*, 1989), where the 10-bp Sp1 consensus is underlined. Since Sp1 recognizes degenerate sequences, and since flanking nucleotides outside of the consensus may also affect the binding affinity (Höller *et al.*, 1988), we decided to also examine another high affinity binding site, 5′-GTGGGGGCGGGGCCTA-3′, from the mouse DHFR gene. As expected, Sp1 was able to bind to this site in a methylation insensitive fashion (Figure 1A).

MTF-1 is a transcription factor essential for basal and metal-induced gene expression of mammalian metallothionein genes, and contains six zinc fingers of the Cys_2-His_2 type (Radtke *et al.*, 1993; Brugnera *et al.*, 1994; Heuchel *et al.*, 1994). The binding site for MTF-1 is called metal responsive element (MRE). Because MREd is the highest affinity site among six MREs (MREa to MREf) found in mouse metallothionein I gene, and confers a particularly high zinc inducibility on heterologous minimal promoters (Stuart *et al.*, 1985; Westin and Schaffner, 1988; Mueller *et al.*, 1988), we tested the effect on MTF-1 binding of methylating MREd. MREd contains two CpG dinucleotides, at the center and at the margin (Figure 1B, bottom). In addition to the binding site for MTF-1, the MREd sequence overlaps with an imperfect recognition site for the transcription factor Sp1. Bandshift experiments under conditions far below saturation, i.e. when only a minor fraction of the labelled oligonucleotide was shifted,

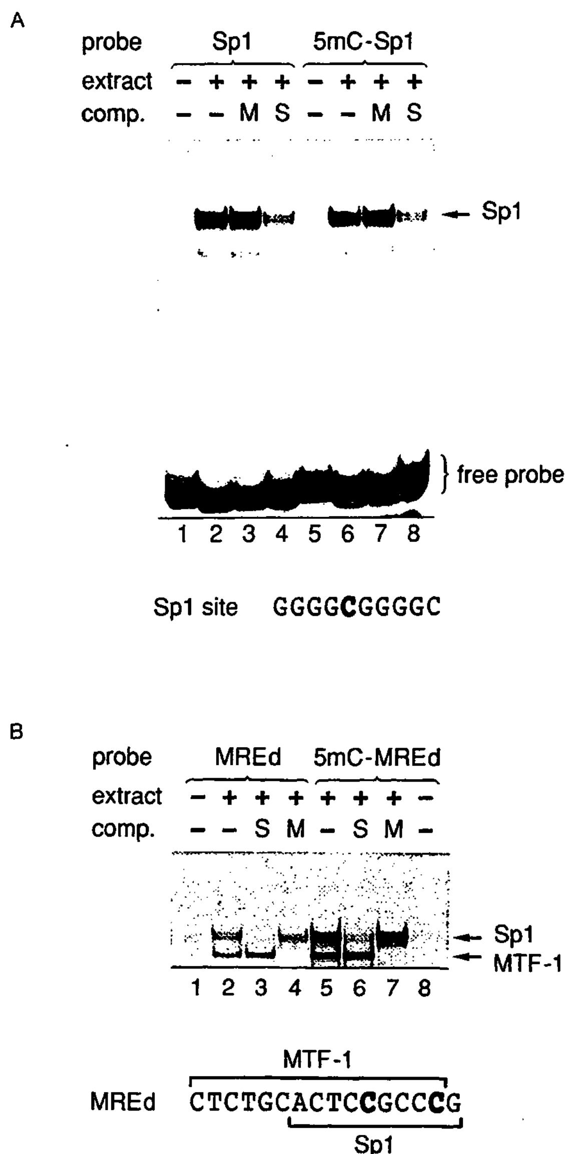


Fig. 1 Binding of MTF-1 and Sp1 to Methylated and Unmethylated Recognition Sites *In Vitro*.

(A) Binding to Sp1 consensus site. Bandshift analysis was performed using nuclear extracts from mouse 3T6 cells. The end-labeled Sp1 site oligonucleotides, unmethylated (Sp1) and methylated (5mC-Sp1), were derived from the mouse DHFR promoter. As competitors (comp.), 200-fold molar excess of unlabeled MRE-s (M) or Sp1HSV (S) oligonucleotide were added prior to addition of extract. The position of the DNA-protein complex is indicated by an arrow labeled Sp1. In this and further Figures, the binding site is shown below, with the methylatable cytosine in bold.

(B) Binding to MREd sequence. Bandshift analysis was performed using nuclear extracts from mouse 3T6 cells. The end-labeled oligonucleotides, unmethylated (MREd) and methylated (5mC-MREd), were derived from the mouse metallothionein-I promoter. Competition was done as above. The overlapping binding sites for MTF-1 and Sp1 are shown below in bold. For comparison to the sequences shown under Figures 1A and 6, the antistrand sequence reads: 5′ CGGGCGGAGTGCAGAG 3′ (Sp1 site underlined). For complete sequence of oligonucleotides containing Sp1 consensus site or MREd or other sites, see Materials and Methods.

Fig. 2 Binding of Krox-20 to Methylated and Unmethylated Recognition Sites *In Vitro*.
Bandshift analysis was performed using bacterial extract containing Krox-20 and control bacterial extract not containing Krox-20 (C). The end-labeled oligonucleotides, unmethylated (Krox) and methylated (5mC-Krox), were derived from the mouse HoxA4 promoter. The competition was done with MRE-s (M) or Krox-20 (K) oligonucleotides as in Figure 1. The position of the DNA-protein complex is indicated by an arrow labeled Krox-20.

showed that MTF-1 as well as Sp1 formed DNA-protein complexes with a methylated MREd probe at an affinity at least as high as with an unmethylated probe (Figure 1B, top). Competition experiments suggested specific binding of these factors. As these two independent zinc finger proteins appeared to be methylation insensitive, we considered the possibility that insensitivity towards methylation might be a general characteristic of Cys_2-His_2 zinc finger proteins. This encouraged us to test a third zinc finger protein, Krox-20.

Krox-20 contains three zinc fingers of Cys_2-His_2 type, which are similar to those of the transcription factor Sp1 (Nardelli *et al.*, 1991). As with Sp1 and MTF-1, bandshift analysis revealed that Krox-20 could also bind to its recognition site irrespective of methylation (Figure 2).

Methylated MREd Can Mediate Zinc Induction

Knowing that MTF-1 is essential for metal-induced transcription of mouse metallothionein genes (Heuchel *et al.*, 1994) and that MTF-1 binds to the methylated MREd (Figure 1B), we examined transcripts from methylated 4×MREd-OVEC, a plasmid carrying four MREd sites upstream of the rabbit β-globin gene, which contained its own TATA box and initiator region. The plasmid 4×MREd-OVEC was methylated *in vitro* at all CpG dinucleotides by the prokaryotic *Sss*I methylase, and transfected into HeLa cells. Completion of the *in vitro* methylation reaction was confirmed by *Hpa*II and *Msp*I digestion and agarose gel electrophoresis (data not shown). As shown by S1 mapping analysis, the methylated promoter was still metal-inducible (Figure 3, left panel), although the overall transcription was lower compared to the mock-methylated control. The cotransfected expression vector for the exogenous mouse MTF-1 had only minor effects on metal inducibility of the methylated 4×MREd-OVEC construct (Figure 3). Similar results were obtained reproducibly with increased amounts of expression vector; thereby, expression of exogenous mouse MTF-1 was verified by electrophoretic mobility shift assay, exploiting the fact that mouse MTF-1 is smaller than the endogenous human MTF-1 of HeLa cells (data not shown). The observed zinc induction strongly suggests that MTF-1 can function on methylated binding sites. The overall reduction in transcription after plasmid methylation was not due to methylation of the MREd sequences but rather an effect of CpG methylation of flanking sequences. This was shown in a further experiment when methylation was confined to the MREd sequences. To this end, we inserted a methylated and, as a control, an unmethylated 4×MREd-oligonucleotide by preparative ligation into the OVEC reporter gene. Ligation products were purified by gel electrophoresis (see Materials and Methods) and directly transfected together with MTF-1 activator gene into dko7 cells. Transfected cells were treated with zinc and reporter gene RNA extracted and analyzed. Despite the methylated MREd binding site, mMTF-1 was still able to activate transcription to 75–93% of the unmethylated control level (Figure 4, lanes 3 and 4; 84%, an average of 3 experiments).

A negative effect of methylation on transcription activity of the natural mouse metallothionein I (MT-I) promoter has been reported by Levine *et al.* (1991), who found that zinc inducibility is abolished when a plasmid containing 1.8 kb of this promoter was methylated by the M. *Sss*I enzyme. We tested another natural metallothionein promoter, the human metallothionein IIA (MT-IIA) promoter, using a β-globin gene as a reporter gene. As is the case for the mouse MT-I promoter, *Sss*I methylation severely reduced both basal and induced transcription from the human MT-IIA promoter (Figure 3, righthand panel, arrow 2). When an MTF-1 expression vector was cotransfected, the initiation site of the β-globin gene, which in this construct was located downstream of the complete human MT-IIA promoter and yielded only marginal transcription initiation, was activated to some extent in a zinc dependent manner (Figure 3, arrow 1, see also Discussion). In conclusion, after methylation the 4×MREd promoter retained its transcriptional activity and good zinc inducibility, while overall transcription from the methylated human MT-IIA promoter was severely repressed.

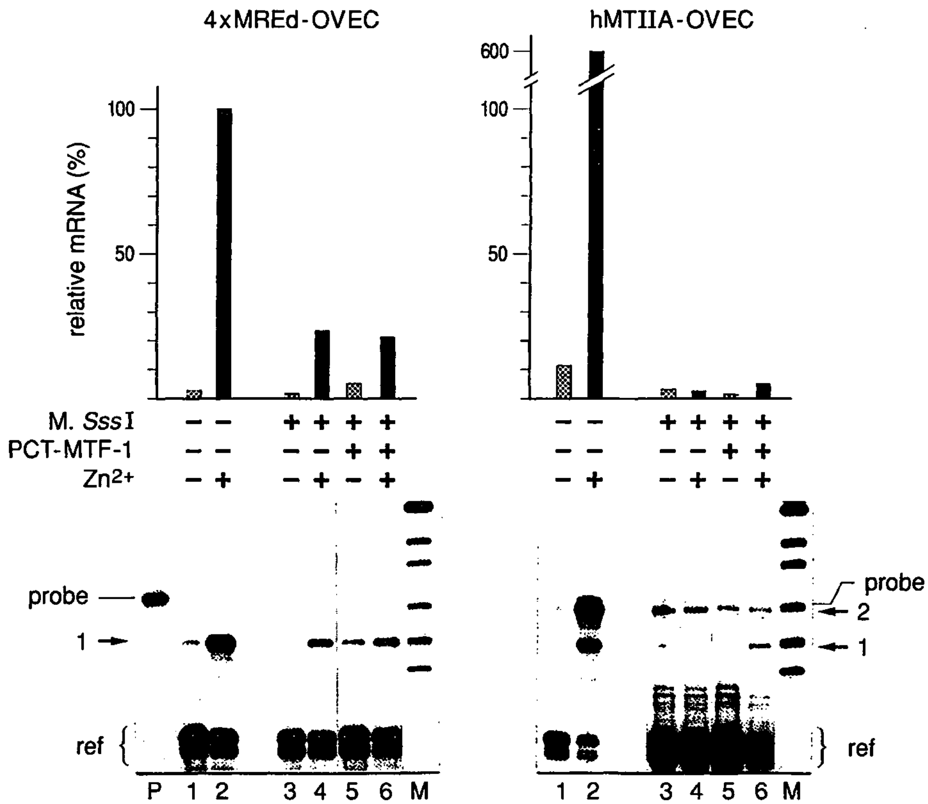


Fig. 3 S1 Nuclease Mapping of RNA from HeLa Cells Transfected with the Reporter Plasmids.
The reporter plasmids, 4×MREd-OVEC and human MT-IIA-OVEC, were methylated (M. *Sss*I +) or mock methylated (M. *Sss*I –) with the *Sss*I methylase. Mouse MTF-1 expression vector PCT-MTF-1 was cotransfected where indicated. Cells were uninduced (–) or induced (+) by zinc treatment. probe: undigested DNA probe; ref: reference transcripts. The arrow 1 indicates transcripts initiated at the β-globin initiator. These are the only transcripts seen with 4×MREd OVEC, and minor transcripts in human MT-IIA OVEC. In the righthand panel, arrow 2 indicates the major transcripts from human MT-IIA start site. Quantitative analysis of the signals is shown by bars above the autoradiograph, whereby the signals of the reporter gene were normalized to the signals from the reference gene. The relative signal of the zinc-stressed HeLa cells transfected with the unmethylated 4×MREd-OVEC was set to 100%. In the righthand panel, signals from band 1 and 2 were pooled for the quantitative analysis. Note that in the lefthand panel, the reduction of overall transcription seen in lanes 3–6 (with methylated 4×MREd-OVEC) versus lanes 1–2 (unmethylated) is an effect of plasmid methylation rather than MREd methylation (see Figure 4). M: *Hpa*II digested pBR322 marker DNA.

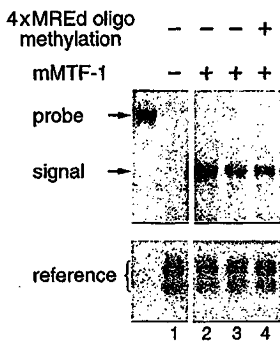


Fig. 4 Transcription of Reporter Plasmids with Inserted Unmethylated or Methylated 4×MREd Oligonucleotide.
RNA preparations from transfected and zinc-induced mouse dko 7 cells were quantified by S1 nuclease mapping. The methylation status of 4×MREd and the presence of the cotransfected expression vector for mouse MTF-1 are indicated above the lanes. Lanes 1 and 2 show the 4×MREd-OVEC vectors grown in *E. coli* (and therefore unmethylated) and lanes 3 and 4 show the 4×MREd-OVEC vectors resulting from insertion of either unmethylated (lane 3) or methylated (lane 4) 4×MREd-oligo. Note that the signals in lanes 2 and 3 in principle are from the same (unmethylated) template DNA; however lane 2 represents a transfection with intact plasmid DNA, while lane 3 represents restricted, ligated and gel-purified DNA that was directly used for transfection, to have a valid comparison to lane 4.

Position of Methyl-CpG within a Metal-Responsive Element (MRE) Differentially Affects Binding of MTF-1

It seemed paradoxical to us that overall *SssI* methylation inhibited the activity of the human MT-IIA promoter more severely than that of the 4×MREd promoter. Since the human MT-IIA promoter contains seven different MRE-related sequences (Stuart *et al.*, 1985), we considered the possibility that the binding of MTF-1 to at least some of these sites was inhibited by methylation. Comparison of the seven MRE-related sequences revealed methylatable CpG sites at a number of positions, namely at nucleotides No. 1, 6, 8, 10, 13, 14 and 15, of which position 6 and 10 are located within the highly conserved 'core' MRE sequence (Figure 5A). Furthermore, these CpG sites at positions 6 and/or 10 within the MRE core are also frequently present in the promoters of human MT-IA, mouse and sheep MT-I, and mouse MT-II genes (Stuart *et al.*, 1985). Therefore we examined more closely the effect of CpG methylation at MRE positions 6 and 10. We first tested MRE-s, a high affinity consensus MRE which contains CpGs at positions 10 and 15. The affinity of both endogenous primate and exogenous mouse MTF-1 for MRE-s was significantly reduced upon methylation (Figure 5B). Since methylation of the CpG at position 15 did not affect binding in the context of MREd (Figure 1B), the observed effect is presumably due to CpG methylation at position 10. Next, we analyzed MREa, which is derived from the mouse MT-I promoter and contains CpGs at positions 6 and 10 (Figure 5A). When this site was methylated binding was almost completely abolished (Figure 5B). We conclude that methylation at position 6, in addition to that at position 10, can interfere with MTF-1 binding. Thus our data suggest that the effect of DNA methylation on MTF-1 binding depends on the relative position of 5-methylcytosines within the binding sites. An alternative explanation would have been that all MRE sites are methylation sensitive, but that our bandshift assay would reveal a difference only with the lower affinity sites, due to saturation of the high affinity site MREd site with MTF-1. This latter possibility can be ruled out, because our bandshift conditions are such that the binding site is in excess and hence not saturated with factors. In addition, the methylated MREd binds to MTF-1 at least as well, if not better, than its unmethylated counterpart (Figure 1B). Interestingly this is also true for Sp1, to a greater or lesser extent depending on the specific binding site (Höller *et al.*, 1988; Figure 1A; see also Jane *et al.*, 1993 and Sengupta *et al.*, 1994).

Discussion

Transcription from 4×MREd and Natural Metallothionein Promoters

CpG methylation seems to inhibit transcription by two mechanisms, firstly by directly preventing the binding of transcription factors, as exemplified by methylation-sen-

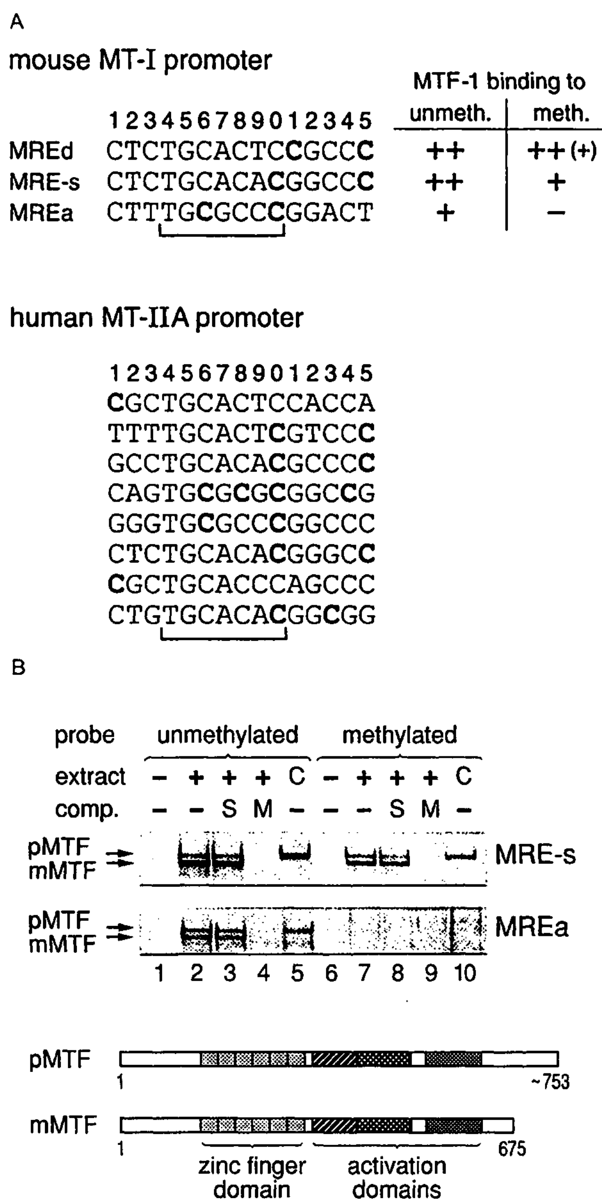


Fig. 5 Structure and Binding Properties of Different MRE Sequences.

(A) Comparison of metal responsive element (MRE) sequences from the mouse MT-I and the human MT-IIA promoters. MREd and MREa are derived from the mouse MT-I promoter (Stuart *et al.*, 1985). MRE-s is a synthetic consensus MRE without an overlapping Sp1 site (Radtke *et al.*, 1993). The results of bandshift experiments are summarized in a table next to the sequences. CpG cytosines are in bold; MRE 'core' nucleotides TGCRCNC are denoted by bracket.

(B) Binding of MTF-1 to methylated and unmethylated recognition sites *in vitro*. Bandshift analysis was performed using nuclear extracts from COS cells alone (C) or from COS cells transfected with cloned mouse MTF-1 (+). As probes, MRE-s or MREa were used. The competition was done as in Figure 1. The DNA-protein complex derived from endogenous MTF-1 in COS cells is indicated by pMTF-1 (primate MTF-1), the one from exogenous mouse MTF-1 by mMTF-1. Shown below is a schematic representation of primate and mouse MTF-1. The primate (monkey) MTF-1 protein has a C-terminal extension of about 78 amino acids compared to the mouse MTF-1 which results in a slightly slower electrophoretic migration (Brugnera *et al.*, 1994).

sitivity of a cyclic AMP-dependent promoter (Iguchi-Ariga and Schaffner, 1989), and secondly by interacting with components inhibitory to transcription, such as histone H1 (Jost and Hofsteenge, 1992; Levine *et al.*, 1993) and/or specialized methyl CpG binding proteins (Wang *et al.*, 1986; Meehan *et al.*, 1989; Boyes and Bird, 1991; Jost and Hofsteenge, 1992; Boyes and Bird, 1992), and/or by inducing inactive chromatin formation on the DNA (Buschhausen *et al.*, 1987; Tazi and Bird, 1990). Our *in vitro* binding experiments and the transfection experiments with reporter plasmids with inserted methylated or unmethylated 4×MREd oligonucleotide demonstrated insensitivity of MTF-1 to MREd methylation. Thus the dramatic inactivation of the human metallothionein IIA promoter by methylation of CpG sites seemed to be best explained by the second, indirect mechanism. (With some constructions not related to the MRE analysis presented here, we indeed found that methylation in the promoter region could contribute to inhibition of transcription even if the transcription factor binding sites were devoid of any CpG; Hug *et al.*, 1996). However, our refined analysis of MTF-1 binding to different MREs later suggested that MTF-1 is sensitive to methylation of MRE positions 6 and 10, offering a straightforward explanation by the direct mechanism; many MRE sites, notably those of MT-IIA, harbor CpG sites at positions 6 and 10 (Figure 5A). An additional contribution to inactivation probably comes from CpGs in the initiator region, even though this region in principle is extremely tolerant of many kinds of sequence alterations (Xu *et al.*, 1991). In fact, transcription from the human MT-IIA initiation region, which harbors several CpG sites, was more strongly inhibited by CpG methylation than that from the 4×MREd-OVEC initiation region, which lacks CpGs and is derived from the β-globin gene (Figure 3 righthand panel, arrows 2 and 1, respectively; see also Murray and Grosveld, 1987).

Possible Role of Methylation Insensitive Zinc Finger Proteins

Two zinc finger proteins, Sp1 and Krox-20, are methylation insensitive, and when tested on their favored binding sites, MTF-1 can bind at leat to a given high affinity site (MREd) irrespective of methylation. These findings may seem paradoxical when we consider the natural context of the binding sites for these factors, which are often found within so-called CpG island promoters (Bird, 1986; Matsuo *et al.*, 1993; and references therein). It is well established that the majority of CpG islands are methylation free in all cell types and developmental stages, some notable exceptions notwithstanding. In other words, transcription factor binding sites for Sp1, Krox-20 or MTF-1 are meant to remain unmethylated when they are located within CpG island promoters. These methylation insensitive factors may help to maintain the methylation free state of CpG islands. Even when *de novo* methylation of CpG island promoter regions occurs accidentally, the transcription factors discussed here may help to (re)establish unmethylated CpGs by remaining bound to the accidentally methylated sites. At least for Sp1, such a role as a demethylation-inducing factor has been postulated before (Höller *et al.*, 1988) and supported by experimental evidence (Macleod *et al.*, 1994; Brandeis *et al.*, 1994; Silke *et al.*, 1995). A biological significance of the methylation insensitivity of factors may become apparent if their binding sites are found within non-CpG island promoters, which are usually methylated during genome-wide *de novo* methylation after the blastocyst stage and remain methylated until around the time when the gene is transcriptionally activated. A transcription factor that binds to a given promoter site irrespective of methylation may then actively counteract the process of CpG methylation. An alternative mechanism has been postulated for γ-globin gene repression by DNA methylation. There, Sp1 factor binds particularly tightly to a methylated promoter site and thus may prevent binding of a stage-specific activator (Jane *et al.*, 1993; Sengupta *et al.*, 1994). Certainly more has to be learned about the mechanisms by which methylation sensitive and insensitive transcription factors/binding sites influence the activity pattern of genes during development.

How Do Sp1 and Krox-20 Bind to Sites Irrespective of CpG Methylation?

According to *in vitro* mutagenesis (Nardelli *et al.*, 1991) and crystallographic studies of the complex between Krox-24/Zif268 and DNA (Pavletich and Pabo, 1991), each zinc finger seems to recognize three adjacent nucleotides of the guanine-rich strand in the major groove. In the simplest scheme, three amino acid residues designated X, Y and Z, corresponding to positions –1, +3, +6 relative to the start of the recognition helix, contact DNA in a one-residue-to-one nucleotide correspondence from the 3′ to the 5′ end of the triplet (Klevit, 1991). This simple scheme may hold for a large number of Cys_2-His_2 type zinc finger proteins (Jacobs, 1992; Choo and Klug, 1994). The first and third fingers of Krox-20 and the second finger of Sp1 have arginine – glutamic acid – arginine at residues X-Y-Z (Figure 6B). Although the two arginines form hydrogen bonds with guanines, the central glutamic acid does not contact the DNA (Pavletich and Pabo, 1991), which may allow these fingers to recognize the triplet $G^{m}CG$ with 5-methylcytosine in the middle. When the central cytosine in the consensus Sp1 binding site 5′-GGGGCGGGG-3′ is replaced by adenine, this variant is still bound by Sp1 with one third of the usual affinity (Letovsky and Dynan, 1989). This is in agreement with the notion that the second finger of Sp1 and the first and third fingers of Krox-20 show flexibility in recognizing the middle position of the triplet DNA, tolerating 5-methylcytosine instead of cytosine at this position. Also, in the case of Krox-20, P. Charnay and colleagues have mutated the central finger to make it similar to finger one or three. In this situation they found very little selectivity for the central nucleotide position, except that G was excluded (Nardelli *et al.*, 1991). This is in agreement with the data of Pavletich and Pabo (1991) who found no

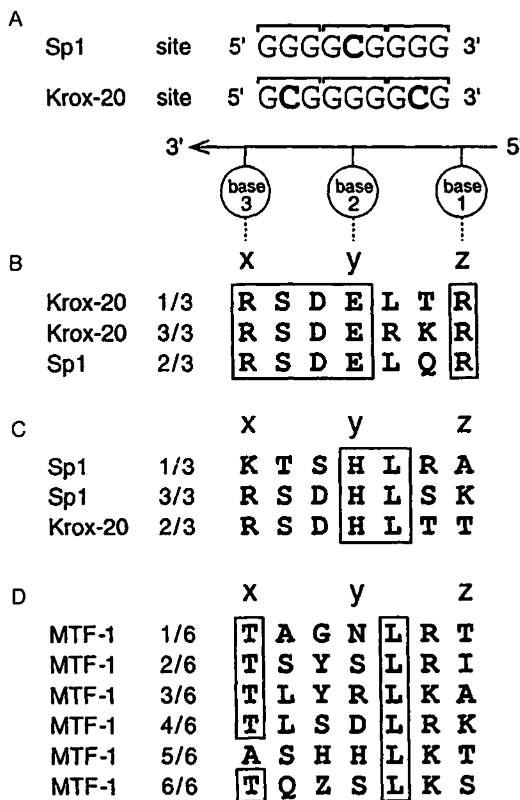


Fig. 6 Possible DNA Base-Amino Acid Contacts in Zinc Fingers. Three residues X, Y and Z, corresponding to positions −1, +3, +6 relative to the start of the recognition helix, thought to be critical for base recognition and neighboring residues. (A) Consensus recognition sequences of Sp1 and Krox-20. The Sp1 site has also been described as **G**/T**G**/A**GGC**/A**GG**/T**G**/A**G**/A**C**/T (Briggs *et al.*, 1986; Ben-Hattar *et al.*, 1989). (B) Zinc fingers of Sp1 and Krox-20 which recognize 5′-**GCG**-3′. (C) Zinc fingers which recognize 5′ **G**/T**GG**-3′. (D) The six zinc fingers of MTF-1. The ratios (e.g. 2/3) indicate the position of the finger and the total number of zinc fingers in the given protein.

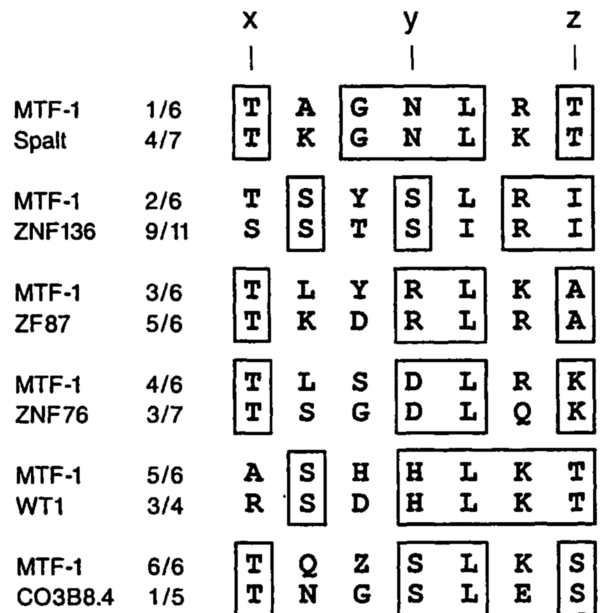


Fig. 7 Comparison of the Six Zinc Fingers of MTF-1 with Other Known Zinc Fingers.
MTF-1 type fingers (X = Thr) are also found in several other zinc finger factors. Three residues X, Y and Z, denote positions −1, +3, +6 relative to the start of the recognition helix, and are thought to be critical for base recognition.

contact at this position; the observed exclusion of G is presumably the result of steric hindrance. Therefore, a cytosine modification at this central position is probably acceptable as long as it is not too voluminous.

Methylation Sensitive and Insensitive Fingers of MTF-1

Our results show that binding of MTF-1 to MRE target sequences can be both methylation sensitive and insensitive, depending on the particular binding site. Cytosines whose methylation impairs MTF-1 binding are conserved among several MRE sequences of the MT-IIA promoter (Figure 5A). They might therefore be directly contacted by a zinc finger. However, an assignment of individual fingers to triplet nucleotides is presently not possible, due to two major problems. Firstly, the consensus MRE is 12 to 15 bp and thus shorter than the predicted 6 × 3 = 18 bp. This may mean that at least one finger is not used for specific DNA binding. Compatible with this idea is our finding that MTF-1 zinc fingers 1 to 3 are more important for MREd binding than fingers 4 to 6 (Radtke *et al.*, 1995). In addition, it is conceivable that MTF-1 might recognize different MRE sequences with different sets of fingers. Secondly, five of the six MTF-1 fingers have Thr at position X (the 5th finger has Ala), whereas in other Cys_2-His_2 zinc fingers including those of Sp1 and Krox-20, Arg or Lys are the two dominant amino acids at position X (Figure 6). Therefore, mutation studies of residues at position Y have been done only with keeping Arg or Lys at position X rather than Thr (Desjarlais and Berg, 1992; 1993). The fact that none of the MTF-1 fingers has Arg at position X nor Z is intriguing since when zinc fingers with randomized amino acids are screened for various triplet specificity, guanine either at the 5′ or 3′ end of a triplet almost always selects fingers with Arg at position Z or X of the α-helix, respectively (Rebar and Pabo, 1994; Choo and Klug, 1994). In order not to depend on guanines at either end of a triplet, we entertain the idea that MTF-1, unlike Sp1 and Krox-20, recognizes the cytosine rich strand. Since the cytosine rich strand can contribute less to the stability/specificity of the protein-DNA interaction in the major groove compared to the guanine rich strand (Choo and Klug, 1994), this would be consistent with the notion that zinc fingers similar to those of MTF-1 are often found in factors with more than three fingers (Figure 7).

Materials and Methods

Construction of Plasmids

The 4×MREd-OVEC reporter plasmid, OVEC-REF reference plasmid and PCTMTF-1 expression vector were described previously

(Westin *et al.*, 1987; Radtke *et al.*, 1993). Briefly, OVEC contains a β-globin reporter gene with its own TATA box and 5′ of it restriction sites for insertion of upstream promoter elements. 4×MREd-OVEC is an OVEC construct with 4 tandem copies of the strong metal-responsive element MREd inserted 5′ of the TATA box. The internal standard OVEC-REF contains the constitutively active enhancer/promoter sequences of SV40. It also has a truncation in the β-globin leader sequence, yielding a faster migrating band in S1 mapping. Thus the reporter and reference gene signals can be obtained with the same S1 DNA probe. To construct hMT-OVEC, the promoter region of the human metallothionein IIA gene (MT-IIA) from −780 to +70 was recovered from pHS1 (Harrington *et al.*, 1988) by *Hin*dIII and *Bam*HI digestion and introduced upstream of the β-globin reporter gene in OVEC-1 (Westin *et al.*, 1987) between *Sac*I and *Pst*I sites by blunt-end ligation.

DNA Transfections and RNA Analysis

HeLa cells were transfected by the calcium phosphate method with 6 μg reporter plasmid, 0.5 μg reference plasmid, and 5 μg of either mouse MTF-1 expression vector or salmon sperm DNA per 10 cm dish as previously described (Radtke *et al.*, 1993). The metal ion induction experiment was done as described (Westin and Schaffner, 1988), with a minor modification, namely the omission of the DMSO shock. In case of the ligation experiment (Figure 4), 0.5 μg reporter plasmid, 0.1 μg reference plasmid, 1 μg of mouse MTF-1 expression vector and 19 μg carrier DNA were transfected into mouse dko7 cells, which are null mutant for MTF-1 as previously described (Heuchel *et al.*, 1994; Radtke *et al.*, 1993). All transfections were done at least twice, with similar results. Transcription signals were measured by PhosphorImager (Molecular Dynamics) and were normalized relative to reference gene transcription. Uncorrected X-ray autoradiograms of the experiments are shown as well to provide raw data.

*Sss*I Methylase and Methylation Reaction

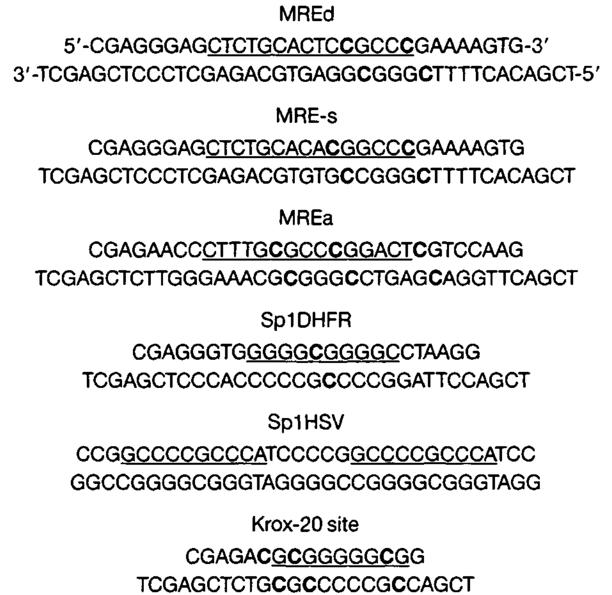
All CpG dinucleotides in the reporter plasmids were methylated for 12 hours at 37 °C using 0.5 U *Sss*I methylase (New England Biolabs) per μg DNA in 10 mM Tris-HCl (pH 7.9); 50 mM NaCl, 1 mM DTT and 10 mM $MgCl_2$. Mock methylation reactions were done by excluding S-adenosylmethionine from the reaction but including *Sss*I methylase. We found that such mock methylation was a better control than omission of *Sss*I methylase, because there was a topoisomerase activity in the *Sss*I methylase preparation that was not affected by the presence or absence of S-adenosylmethionine (Matsuo *et al.*, 1994). Completeness of the methylation reaction was checked by *Hpa*II digestion of methylated and mock-methylated plasmids.

Ligation of Oligonucleotide into the Vector Backbone

In a large scale ligation, the 4×MREd oligonucleotide, either methylated or unmethylated, was ligated into OVEC. We used vectors and oligonucleotides with overhangs generated by the restriction enzymes *Sfi*I and *Ava*I for the ligation. Their non-palindromic recognition sequences reduced the possibility of incorrect ligation products and increased the yield of the desired ligation product. Both closed circular and nicked circular forms were produced by the ligation reaction and tested after separation of the two forms by preparative agarose gel electrophoresis.

Nuclear Extract Preparation and Bandshift Analysis

Nuclear extracts from COS cells transfected with 8 μg of PCT-MTF-1, and from 3T6 cells, were prepared as previously described (Schreiber *et al.*, 1989). Bacterial extract containing Krox-20 was a gift of Patrick Charnay (Paris). For the bandshift experiments the following double stranded oligonucleotide probes and competitors were used (CpG cytosines in bold):


MREd
5′-CGAGGGAGCTCTGCACTCCGCCCGAAAAGTG-3′
3′-TCGAGCTCCCTCGAGACGTGAGGCGGGCTTTTCACAGCT-5′
MRE-s
CGAGGGAGCTCTGCACACGGCCCGAAAAGTG
TCGAGCTCCCTCGAGACGTGTGCCGGGCTTTTCACAGCT
MREa
CGAGAACCCTTTGCGCCCGGACTCGTCCAAG
TCGAGCTCTTGGGAAACGCGGGCCTGAGCAGGTTCAGCT
Sp1DHFR
CGAGGGTGGGGGCGGGGCCTAAGG
TCGAGCTCCCACCCCCGCCCCGGATTCCAGCT
Sp1HSV
CCGGCCCCGCCCATCCCCGGCCCCGCCCATCC
GGCCGGGGCGGGTAGGGGCCGGGGCGGGTAGG
Krox-20 site
CGAGACGCGGGGGCGG
TCGAGCTCTGCGCCCCCGCCAGCT

MREd is derived from the mouse metallothionein I promoter; MRE-s is a synthetic MRE consensus sequence; MREa is derived from the mouse metallothionein I promoter; Sp1DHFR and Sp1HSV, GC boxes from the mouse dihydrofolate reductase (DHFR) gene and from the immediate-early 3 gene of herpes simplex virus, respectively; Krox-20 site, from the mouse HoxA4 promoter (Chavrier *et al.*, 1988). Underlined nucleotides indicate DNA recognition sites for MTF-1, Sp1 and Krox-20. Highlighted cytosines indicate positions of 5-methylcytosines incorporated into methylated DNA oligonnucleotides during synthesis. 6 fmoles of probe end-labelled with ^{32}P γ-ATP (between 15′000 and 20′000 cpm per assay) were incubated with 5 μg of nuclear extract. The composition of the incubation buffer was 12 mM Hepes pH 7.9, 12% glycerol, 5 mM NaCl, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.6 mM DTT and 100 mM $ZnSO_4$. To confirm the specificity of binding, 2 pmoles of a specific competitor or unspecific competitor were added to the reaction as indicated. All binding studies were done at least twice, with similar results. DNA-protein binding reactions were performed as previously described (Vesque and Charnay, 1992; Radtke *et al.*, 1993).

Acknowledgements

We are indebted to Dr. Patrick Charnay (Paris) for a gift of the bacterial extract containing Krox-20 and the control extract, and to Drs. Patrick Charnay and Rainer Heuchel for valuable discussions. We also thank Drs. Chris Hovens, Philippe Douville, John Silke and Oliver Clay for critical reading of the manuscript and Mr. Fritz Ochsenbein for preparing the figures.

References

Ben-Hattar, J., Beard, P., and Jiricny, J. (1989). Cytosine methylation in CTF and Sp1 recognition sites of an HSV tk promoter: effects on transcription *in vivo* and on factor binding *in vitro*. Nucl. Acids Res. *17*, 10179–10190.

Bestor, T.H., and Ingram, V.M. (1983). Two DNA methyltransferases from murine erythroleukemia cells: purification, sequence

specificity, and mode of interaction with DNA. Proc. Natl. Acad. Sci. USA *80*, 5559–5563.

Bird, A. (1986). CpG-rich islands and the function of DNA methylation. Nature *321*, 209–213.

Boyes, J., and Bird, A. (1991). DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. Cell *64*, 1123–1134.

Boyes, J., and Bird, A. (1992). Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein. EMBO J. *11*, 327–333.

Brandeis, M., Frank, D., Keshet, I., Siegfried, Z., Mendelsohn, M., Nemes, A., Temper, V., Razin, A., and Cedar, H. (1994). Sp1 elements protect a CpG island from *de novo* methylation. Nature *371*, 435–438.

Briggs, M.R., Kadonaga, J.T., Bell, S.P., and Tjian, R. (1986). Purification and biochemical characterization of the promoter-specific transcription factor Sp1. Science *234*, 47–52.

Brugnera, E., Georgiev, O., Radtke, F., Heuchel, R., Baker, E., Sutherland, G.R., and Schaffner, W. (1994). Cloning, chromosomal mapping and characterization of the human metal-regulatory transcription factor MTF-1. Nucl. Acids Res. *22*, 3167–3173.

Buschhausen, G., Wittig, B., Graessmann, M., and Graessmann, A. (1987). Chromatin structure is required to block transcription of the methylated herpes simplex virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA *84*, 1177–1181.

Chavrier, P., Zerial, M., Lemaire, P., Almendral, J., Bravo, R., and Charnay, P. (1988). A gene encoding a protein with zinc fingers is activated during G0/G1 transition in cultured cells. EMBO J. *7*, 29–35.

Christy, B.A., Lau, L.F., and Nathans, D. (1988). A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc. Natl. Acad. Sci. USA *85*, 7857–7861.

Choo, Y., and Klug, A. (1994). Towards a code for the interactions of zinc fingers with DNA: selection of randomized fingers displayed on phage. Proc. Natl. Acad. Sci. USA *91*, 11163–11167.

Courey, A.J., and Tjian, R. (1992). Mechanisms of transcriptional control as revealed by studies of human transcription factor Sp1. Transcriptional Regulation. In S.L. McKnight and K.R. Yamamoto, eds. (Cold Spring Harbor, N.Y., USA: Cold Spring Harbor Laboratory Press), Book 2, pp. 743–769.

Desjarlais, J.R., and Berg, J.M. (1992). Towards rules relating zinc finger protein sequences and DNA binding site preferences. Proc. Natl. Acad. Sci. USA *89*, 7345–7349.

Desjarlais, J.R., and Berg, J.M. (1993). Use of a zinc-finger consensus sequence framework and specificity rules to design specific DNA binding proteins. Proc. Natl. Acad. Sci. USA *90*, 2256–2260.

Ehrlich, M., and Ehrlich, K.C. (1993). Effect of DNA methylation on the binding of vertebrate and plant proteins to DNA. In DNA Methylation: Molecular Biology and Biological Significance. J.-P. Jost and H.P. Saluz, eds., (Basel, Switzerland: Birkhäuser Verlag), pp. 145–168.

Harrington, M.A., Jones, P.A., Imagawa, M., and Karin, M. (1988). Cytosine methylation does not affect binding of transcription factor Sp1. Proc. Natl. Acad. Sci. USA *85*, 2066–2070.

Hergersberg, M. (1991). Biological aspects of cytosine methylation in eukaryotic cells. Experientia *47*, 1171–1185.

Heuchel, R., Radtke, F., Georgiev, O., Stark, G., Aguet, M., and Schaffner, W. (1994). The transcription factor MTF-1 is essential for basal and heavy metal-induced metallothionein gene expression. EMBO J. *13*, 2870–2875.

Höller, M., Westin, G., Jiricny, J., and Schaffner, W. (1988). Sp1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated. Genes Dev. *2*, 1127–1135.

Hug, M., Silke, J., Georgiev, O., Rusconi, S., Schaffner, W., and Matsuo, K. (1996). Transcriptional repression by methylation: cooperativity between a CpG cluster in the promoter and remote CpG-rich regions. FEBS Letters, in press.

Iguchi-Ariga, S.M.M., and Schaffner, W. (1989). CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation. Genes Dev. *3*, 612–619.

Jacobs, G.H. (1992). Determination of the base recognition positions of zinc fingers from sequence analysis. EMBO J. *11*, 4507–4517.

Jane, S.M., Gumucio, D.L., Ney, P.A., Cunningham, J.M., and Nienhuis, A.W. (1993). Methylation-enhanced binding of Sp1 to the stage selector element of the human gamma-globin gene promoter may regulate development specificity of expression. Mol. Cell. Biol. *13*, 3272–3281.

Joel, P., Shao, W., and Pratt, K. (1993). A nuclear protein with enhanced binding to methylated Sp1 sites in the AIDS virus promoter. Nucleic Acids Res. *21*, 5786–5793.

Jost, J.-P., and Hofsteenge, J. (1992). The repressor MDBP-2 is a member of the histone H1 family that binds preferentially *in vitro* and *in vivo* to methylated nonspecific DNA sequences. Proc. Natl. Acad. Sci. USA *89*, 9499–9503.

Kadonaga, J., Carner, K.R., Maslarz, F.R., and Tjian, R. (1987). Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell *51*, 1079–1090.

Kaptein, R. (1991). Zinc-finger structures. Curr. Opion. Struct. Biol. *2*, 109–115.

Klevit, R.E. (1991). Recognition of DNA by Cys_2, His_2 zinc fingers. Science *253*, 1367, 1393.

Kovesdi, I., Reichel, R., and Nevins, J.R. (1987). Role of an adenovirus *E2* promoter binding factor in E1A-mediated coordinate gene control. Proc. Natl. Acad. Sci. USA *84*, 2180–2184.

Lemaire, P., Revelant, O., Bravo, R., and Charnay, P. (1988). Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. Proc. Natl. Acad. Sci. USA. *85*, 4691–4695.

Leonhardt, H., and Bestor, T.H. (1993). Structure, function and regulation of mammalian DNA methyltransferase. In DNA Methylation: Molecular Biology and Biological Significance. J.-P. Jost and H.P. Saluz, eds. (Basel, Switzerland: Birkhäuser Verlag), pp. 109–119.

Letovsky, J., and Dynan, W.S. (1989). Measurement of the binding of transcription factor Sp1 to a single GC box recognition sequence. Nucl. Acids Res. *17*, 2639–2653.

Levine, A., Cantoni, G.L., and Razin, A. (1991). Inhibition of promoter activity by methylation: possible involvement of protein mediators. Proc. Natl. Acad. Sci. USA *88*, 6515–6518.

Levine, A., Yeivin, A., Ben-Asher, E., Aloni, Y., and Razin, A. (1993). Histone H1-mediated inhibition of transcription initiation of methylated templates *in vitro*. J. Biol. Chem. *268*, 21754–21759.

Macleod, D., Charlton, J., Mullins, J., and Bird, A.P. (1994). Sp1 sites in the mouse *aprt* gene promoter are required to prevent methylation of the CpG island. Genes Dev. *8*, 2282–2292.

Matsuo, K., Clay, O., Takahashi, T., Silke, J., and Schaffner, W. (1993). Evidence for erosion of mouse CpG islands during mammalian evolution. Som. Cell Mol. Genet. *19*, 543–555.

Matsuo, K., Silke, J., Gramatikoff, K., and Schaffner, W. (1994). The CpG-specific methylase *SssI* has topoisomerase activity in the presence of Mg^{2+}. Nucleic Acids Res. *22*, 5354–5359.

Meehan, R.R., Lewis, J.D., McKay, S., Kleiner, E.L., and Bird, A.P. (1989). Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. Cell *58*, 499–507.

Mueller, P.R., Salser, S.J., and Wold, B. (1988). Constitutive and metal-inducible protein: DNA interactions at the mouse metallothionein I promoter examined by in vivo and in vitro footprinting. Genes Dev. *2*, 412–427.

Murray, E.J., and Grosveld, F. (1987). Site specific demethylation in the promoter of human γ-globin gene does not alleviate methylation mediated suppression. EMBO J. *6*, 2329–2335.

Nardelli, J., Gibson, T.J., Vesque, C., and Charnay, P. (1991). Base sequence discrimination by zinc-finger DNA-binding domains. Nature *349*, 175–178.

Pavletich, N.P., and Pabo, C.O. (1991). Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1Å. Science *252*, 809–817.

Radtke, F., Heuchel, R., Georgiev, O., Hergersberg, M., Gariglio, M., Dembic, Z., and Schaffner, W. (1993). Cloned transcription factor MTF-1 activates the mouse metallothionein I promoter. EMBO J. *12*, 1355–1362.

Radtke, F., Georgiev, O., Müller, H.-P., Brugnera, E., and Schaffner, W. (1995). Functional domains of the heavy metal-responsive transcription regulator MTF-1. Nucleic Acids Res. *23*, 2277–2286.

Rebar, E., and Pabo, C. (1994). Zinc finger phage: affinity selection of fingers with new DNA-binding specificities. Science *263*, 671–673.

Schreiber, E., Matthias, P., Müller, M.M., and Schaffner, W. (1989). Rapid detection of octamer binding proteins with "mini-extracts", prepared from a small number of cells. Nucleic Acids Res. *17*, 6419.

Sengupta, P.K., Lavelle, D., and De Simone, J. (1994). Increased binding of Sp1 to the gamma-globin gene promoter upon site-specific cytosine methylation. Am. J. Hematol. *46*, 169–172.

Sham, M.H., Vesque, C., Nonchev, S., Marshall, H., Frain, M., Das Gupta, R., Whiting, J., Wilkinson, D., Charnay, P., and Krumlauf, R. (1993). The zinc finger gene Krox 20 regulates HoxB2 (Hox2.8) during hindbrain segmentation. Cell *72*, 183–196.

Silke, J., Rother, K.I., Georgiev, O., Schaffner, W., and Matsuo, K. (1995). Complex demethylation patterns at Sp1 binding sites in F9 embryonal carcinoma cells. FEBS letters *370*, 170–174.

Simon, D., Stuhlmann, H., Jahner, D., Wagner, H., Werner, E., and Jaenisch, R. (1983). Retrovirus genomes methylated by mammalian but not bacterial methylase are non-infectious. Nature *304*, 275–277.

Stuart, G.W., Searle, P.F., and Palmiter, R.D. (1985). Identification of multiple metal regulatory elements in mouse metallothionein-I promoter by assaying synthetic sequences. Nature *317*, 828–831.

Tazi, J., and Bird, A. (1990). Alternative chromatin structure at CpG islands. Cell *60*, 909–920.

Vesque, C., and Charnay, P. (1992). Mapping functional regions of the segment-specific transcription factor Krox-20. Nucl. Acids Res. *20*, 2485–2492.

Wang, R.Y., Zhang, X.Y., Khan, R., Zhou, Y.W., Huang, L.H., and Ehrlich, M. (1986). Methylated DNA-binding protein from human placenta recognizes specific methylated sites on several prokaryotic DNAs. Nucleic Acids Res. *14*, 9843–9860.

Westin, G., Gerster, T., Müller, M.M., Schaffner, G., and Schaffner, W. (1987). OVEC, a versatile system to study transcription in mammalian cells and cell-free extracts. Nucl. Acids Res. *15*, 6787–6798.

Westin, G., and Schaffner, W. (1988). A zinc-responsive factor interacts with a metal-regulated enhancer element (MRE) of the mouse metallothionein-I gene. EMBO J. *7*, 3763–3770.

Xu, L., Thali, M., and Schaffner, W. (1991). Upstream box/TATA box order is the major determinant of the direction of transcription. Nucl. Acids Res. *19*, 6699–6704.

Received September 15, 1995; accepted November 29, 1995