Constitutive and metal-inducible protein:DNA interactions at the mouse metallothionein I promoter examined by in vivo and in vitro footprinting

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A method of high resolution in vivo footprinting has been developed and used to survey the mouse metallothionein I (MT-I) promoter for protein : DNA interactions associated with basal-level transcription and with high-level metal-induced transcription. This promoter and its associated regulatory region is structurally complex. It contains multiple potential binding sites for metal regulatory factors and for other transcription factors, including SP1 and MLTF. In several cases potential recognition sites overlap, and the experiments reported here provide a view of which sites are utilized in vivo. These data also show how the pattern of protein : DNA contacts changes when cells are shifted from basal-level expression to metal-induced expression. The noninduced footprint pattern consists of interactions at basal elements that are thought to be responsible for the moderate transcription of this gene in the absence of added metals. These interactions remain unchanged upon metal induction. When MT-I expression is increased by exposing cells to zinc or cadmium, a new footprint pattern is observed. It includes the basal interactions and a new set of metal-dependent footprints that are positioned over all five genetically defined metal responsive elements (MREs), MRE-A-MRE-E. In addition, these data identify a sixth probable MRE, MRE-F, which displays a dimethylsulfate (DMS) footprint similar to that at other MREs.

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The structure of most eukaryotic promoters and associated regulatory regions is complex, consisting of an array of multiple, interdigitated sequence elements that act as recognition sites for the proteins that govern transcription [Dynan and Tjian 1985; reviewed by Maniatis et al. 1987]. The task of identifying physiologically relevant sites and dissecting their individual functions is complicated by several factors: Functional binding sites are not readily discriminated from nonfunctional sites by primary sequence alone; a given segment of DNA sometimes serves as a binding site for two or more different factors; and it is often difficult to generate mutants that ablate all copies of a recognition site without also distorting the remaining sequences and their spatial relationships. In vivo footprinting studies can circumvent some of these problems by providing information on when and how recognition sites are occupied in the cell [Ephrussi et al. 1985; Giniger et al. 1985; Jackson and Felsenfeld 1985; Zinn and Maniatis 1986; Kemper et al. 1987]. In this work we use in vivo footprinting of the mouse metallothionein-I (MT-I) promoter to begin to characterize DNA : protein contacts as they occur in the cell under noninducing and inducing conditions.

Metallothionein genes (MT) code for small, cysteine-rich proteins that bind heavy metals, and they are present in all higher eukaryotes surveyed [reviewed by Karin 1985; Hamer 1986]. Expression of the murine MT-I gene is regulated in response to several different physiological cues. Mouse MT-I is typically expressed in cultured cells at a significant basal level of about 10^2 mRNAs per cell [P. Mueller, unpubl.], in the presence of added zinc or cadmium the mRNA level increases 5- to 20-fold. This metal-dependent accumulation of MT-I RNA is primarily due to increased transcription [Durnam and Palmiter 1981; Hamer and Walling 1982]. A segment of the gene containing the promoter and flanking sequences has been shown to direct both basal and induced transcription of adjacent heterologous sequences [Brinster 1982; Mayo et al. 1982, Pavlakis and Hamer 1983]. DNA transfection studies of deletion mutations and synthetic promoters, together with DNA sequence data from many MT genes, have led to the identification of sequence elements that are responsible for metal induction (metal responsive elements or MREs) [Carter et al. 1984; Stuart et al. 1984, 1985; Searle et al. 1985]. Other sequence elements influence the levels of
both basal and induced expression. In vitro transcription of the related human MT-IIA gene showed that basal transcription can be increased by the addition of SP1 [Lee et al. 1987a] and other factors including AP1 and AP2 [Lee et al. 1987b; Mitchell et al. 1987], but metal-dependent transcription has not yet been established in vitro, and metal dependent in vitro footprints have been confined to a single cadmium-specific site near MRE-D [Seguin and Hamer 1987].

The in vivo footprints presented here define two patterns of apparent protein : DNA contacts associated with basal level transcription and metal induced transcription of the murine MT-I gene. The footprinting strategy uses cells containing transfected, amplified MT genes to increase sensitivity and a genomic primer extension reaction to provide high resolution footprints. Our observations, taken together with existing genetic data, suggest that proteins bound constitutively at basal elements support transcription in the absence of metal induction, and also amplify the increase in transcription mediated by MREs in the presence of metals. These results focus attention on the potential for in vivo competition among factors at overlapping recognition sites, and also provide an explicit set of predictions for DNA-binding patterns expected from in vitro studies.

Results

In vivo footprinting strategy

In this work two different methods have been used to visualize in vivo protein : DNA contacts on the mouse MT-I promoter. The highest-resolution picture of these interactions was obtained by treating intact cells with dimethylsulfate (DMS). DMS methylates guanine residues at the N7 position, rendering them susceptible to subsequent cleavage with piperidine [Maxam and Gilbert 1980]. Cellular proteins bound at or near specific guanines can either enhance or reduce the frequency of DMS methylation relative to the same residues in naked DNA [Gilbert et al. 1976]. Because cell membranes are freely and rapidly permeable to DMS, this experiment permits detection of DNA : protein contacts in intact cells that have undergone a minimum of physiological disruption. DMS was originally used for this purpose by Giniger et al. [1985] and Ephrussi et al. [1985], and in the work presented here the procedure was adapted for mouse L cells.

The second method used to detect protein : DNA interactions is DNase I treatment of nuclei. Because DNase I is a large molecule and does not freely cross the cell membrane, cells must be lysed to expose nuclei for digestion. Although such nuclei are probably not initiating new rounds of transcription [Groudine et al. 1981], it is clear from prior DNase studies of several different promoters that many specific protein : DNA contacts can be detected [Jackson and Felsenfeld 1985, 1987; Zinn and Maniatis 1986; Gimble and Max 1987]. Moreover, DNase I footprints identify protein : DNA interactions at all four residues. Therefore we use this method to complement data obtained by DMS treatment.

DNase and DMS footprints were visualized using a modification of the genomic sequencing procedure of Huibregtse and Engelke (1986). Cleaved genomic DNA was hybridized with a vast excess of a specific, end-labeled oligonucleotide primer under conditions that minimize self-renaturation of genomic DNA. The majority of the unhybridized primer was removed, and the bound primer was extended with AMV reverse transcriptase. Finally, the extension products were separated on a sequencing gel.

The in vivo footprint experiments were performed on L-cell lines transfected with a mouse MT-I–dihydrofolate reductase (DHFR) fusion gene. The gene contains 1775 bp of the MT-I promoter and 5′-flanking sequence fused to the coding region of DHFR cDNA (Fig. 1a). This segment of the MT-I gene has been shown to direct both basal and metal-induced gene expression when transfected into cultured cells or mouse eggs [Brinster et al. 1982; Mayo et al. 1982; Pavlakis and Hamer 1983]. The cell lines used here, A-0.3 and A-60, were selected because they contain multiple copies of the fusion gene, thus increasing the sensitivity of the footprint experiments. Expression of the fusion genes follows the pattern expected for MT-I regulated genes (Fig. 1b and P. Mueller, S. Salser, and B. Wold, unpubl.). In the absence of added metals, A-60 and A-0.3 cell lines display characteristic basal-level expression; upon stimulation with zinc this level increased by 5- and 10-fold, respectively.

A DMS footprint of the MT-I promoter in A-60 cells under both noninducing and inducing conditions is shown in Figure 2. Pairwise comparison of in vitro DMS-treated DNA, {‘naked’ DNA, Fig. 2, lanes 5 and 10} with companion in vivo DMS-treated samples from cells grown in the presence or absence of heavy metal induction [Fig. 2, + zinc, lanes 4 and 9; no zinc, lanes 3 and 8] reveals several footprints in the region between −35 and −190, and these are discussed individually in the sections that follow. By contrast, there are no detectable interactions between −30 and +20, except a single, small, but reproducible, hypersensitivity localized just before the transcription start site. The 25-base oligonucleotides used to prime reverse transcription of cleavage products are complementary to residues −227 to −203 on the noncoding strand and to residues +47 to +23 on the coding strand. With these primers we can visualize both strands of the metal-responsive promoter sequence, as defined previously in studies of 5′ and 3′ deletion mutations [Carter et al. 1984; Stuart et al. 1984; Searle et al. 1985]. Footprint experiments were performed several times on both cell lines, and DNA preparations from these DMS treatments were often assayed in multiple independent footprints. All interactions detected were highly reproducible. Densitometric traces of the autoradiograms were made, and the results are summarized in Figure 3, in which the height of the arrows is proportional to the degree of protection or enhancement at a given site.

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Figure 1. Zinc induction of MT/DHFR. (a) Map of plasmid MD which contains -7458 bp. Thin lines are pBR322, thick solid lines are mouse MT-I, and thick striped lines are mouse DHFR. Construction is described in Materials and methods. (b) MT-I/DHFR RNA measurements. RNA (15 μg total was hybridized with a uniformly labeled, 126-base riboprobe that was complementary to 80 bases (---~) of the MT-promoted DHFR mRNA as per Zinn et al. [1983] and Material and methods. The endogenous DHFR is complementary to 65 bases of this riboprobe, but can not be seen on these short exposures (~30 min). [Lanes 1-3], A-0.3 RNA; [lane 1], without zinc; [lane 2] 130 mM zinc sulfate for 4 hr; [lane 3] 130 mM zinc sulfate for 8 hr. [Lanes 4-9] A-60 RNA; [lane 4] without zinc; [lane 5] 130 mM zinc sulfate for 4 hr; [lane 6] 130 mM zinc sulfate for 8 hr; [lane 7] same as lane 4; [lane 8], 0.5% serum for total of 36 hr; [lane 9], 0.5% serum for total of 36 hr and 60 ng/ml TPA for the last 12 hr.

Figure 2. (a) In vivo DMS footprinting of the mouse MT-I promoter. Noncoding and coding strands are visualized by primer extension. The first and second loadings of the reaction products on a 60-cm gel are placed on top of each other with a slight space between them. Distance from the start of transcription (TXN) is shown to the left of each strand with '-' indicating 5' from start and '-' indicating 3' from start. Potential sites for binding of transcription factors [based on DNA sequence and genetic data, see text] are shown by brackets to the right of each strand. A-60 cells were the source of the DNAs. [DMS] dimethylsulfate; [PIP] piperidine; [NA] naked DNA; [−ZN], DNA methylated in vivo from noninduced cells; [+ZN] DNA methylated in vivo from zinc-induced cells. [Lanes 1 and 5] Control samples containing in vitro purified [naked] DNA that was not treated with DMS or piperidine. [Lanes 2 and 6] Control samples containing in vitro purified [naked] DNA that was only treated with piperidine. [Lanes 3 and 7] DNA from noninduced cells treated with DMS in vivo and piperidine in vitro. [Lanes 4 and 9] DNA from cells induced with 130 mM zinc sulfate for 4 hr before being treated with DMS in vivo and piperidine in vitro. [Lanes 5 and 10] In vitro purified DNA [naked] treated with DMS and piperidine in vitro. See Materials and methods for experimental details and Figure 3 for summary of data. (b) Detail of SP1-A coding strand footprint. [Enlarged inset] Shorter exposure of coding strand over SP1-A region enlarged to show detail and constitutive nature of the SP1 in vivo footprint. [NA] Naked DNA methylated in vivo; [−ZN] DNA methylated in vivo from noninduced cells; [+ZN] DNA methylated in vivo from zinc-induced cells. Vertical lines beneath photo identify guanines in sequence. Densitometry plots show the initial, normalized densitometry traces of the same region enlarged above, and are superimposed on each other as indicated. Difference plots illustrate regions of footprinting in noninduced and induced cells, and were obtained by subtracting the superimposed densitometry plots from each other as indicated. Horizontal baseline represents zero difference or no footprint; traces below it represent hypersensitivity; and traces above it represent protection. These data are quantitated in Figure 3.
Figure 2. (See facing page for legend.)
Mueller et al. protection at the fifth and tenth bases. Specific guanine residues are protected from DMS attack when those residues are closely associated with a protein. If binding of a particular protein results in complete exclusion of DMS from guanines in its recognition site, 100% protection will be observed when all copies of that site are occupied continuously. Guanines –184 to –187 of the SP1-A site are 70% protected in vivo, suggesting that at least 70% of the MT-I genes are occupied throughout the 2-min DMS treatment or, alternatively, all copies in all cells are occupied 70% of the time on average. In contrast to protection from DMS attack, quantitation of hypersensitivity cannot be interpreted in a similarly straightforward fashion. It is thought that hypersensitivity to DMS

![Diagram of mouse MT-I promoter footprints](image)

**Figure 3.** Summary of in vivo DMS and DNase footprinting over the mouse MT-I promoter. The sequence of both strands of the mouse MT-I promoter are shown. [N-CD] Noncoding strand; [CD] coding strand. Base pairs –200 through –91 are in the top half of the figure, and base pairs –90 through +20 are in the bottom half of the figure. Potential recognition sites are shown schematically between the noninduced and induced sequences. These sites are also localized directly on the sequences by horizontal lines: (SP1 and MLTF) a line over the coding strand; (MRE) a line between the strands; (G element) a line below the noncoding strand; (TATA box) a box around both strands. Changes in methylation patterns (Fig. 2) are shown directly over the coding strand and under the noncoding strand; protections are indicated by downward pointing arrows (↓), and hypersensitivities are indicated by upward pointing arrows (↑). See the scale at the bottom of the figure for quantitation. All protections ≥15% and hypersensitivities >1.3-fold are shown. Regions of protection from DNase digestion under noninduced conditions [Fig. 4] are shown for both strands by a set of thick double lines above the noninduced DMS footprints. The sequence of this promoter is as per Glanville et al. (1981), except that an additional adenine was found at –112 (S.J. Salser, unpubl.) and is added to the sequence and numbering throughout this paper.
results when protein : DNA interactions create a local hydrophobic environment around a target guanine [Gilbert et al. 1976]. It is also possible that local changes in DNA conformation might alter DMS sensitivity [Johnsrud 1978]. Given the nature of hypersensitivity, it is not possible to deduce the degree of occupancy by comparison with DMS-treated naked DNA, but it is informative to compare hypersensitivity at a given site under noninducing and inducing conditions.

In agreement with the DMS data, the SP1-A site in MT-I is protected from DNase I digestion of nuclei, indicating that protein is bound at this site. A DNase I footprint of A-0.3 nuclei is shown in Figure 4. Comparison of the DNase I pattern on naked DNA (Fig. 4, lanes 2 and 6) and on nuclei of noninduced cells (Fig. 4, lanes 4 and 8) reveals a protected region of about 20 nucleotides at the SP1-A site in nuclei. This pattern of DNase protection is in agreement with the pattern observed for SP1 binding to the SV40 promoter in vitro (Gidoni et al. 1984, 1985; and see below) and in vivo [Buchanan and Gralla 1987].

The second prospective SP1 site, SP1-B, is centered at -139. It is a 9 of 10 match with the consensus element, includes a perfect match to the GCCGGG core, and is inverted in orientation relative to the site at -182. A DMS footprint is observed at this site, but it is much less intense than the one at the SP1-A site, and the pattern of protection and hypersensitivity is quite different from the SP1 DMS consensus pattern defined in vitro (Gidoni et al. 1984; Jones et al. 1986) and observed for the SP1-A site in vivo. The DNase I experiment corroborates the DMS data, showing a region of protection surrounding the -139 sequence (Fig. 4).

The data presented above suggest that the site at -182 is a bonafide SP1 site that is occupied in mouse L cells. The proximal site, on the other hand, shows an in vivo footprint that bears little resemblance to a simple SP1 interaction. Although the footprint at the B site does not look like a typical SP1 interaction, it remains possible that in the living cell multiple factors, including SP1, compete for this site. To determine directly if, in the absence of competing factors, bona fide SP1 binds anywhere on the MT-I promoter, an in vitro footprint experiment was done using purified SP1 [a gift of J. Kadonaga and R. Tjian.]. Varying amounts of homogeneous

Figure 4. In vivo DNase I footprinting of the mouse MT-I promoter. The MT promoter was footprinted using DNase I as a cleaving agent as in Zinn and Maniatis [1986] and the footprint was detected by primer extension [see Fig. 2 legend and Materials and methods]. The noncoding strand is shown on the right and the coding strand is shown on the left, and potential factor binding sites are indicated by brackets next to the photo. Regions of DNase protection are shown by the thick vertical lines to the left of the brackets. These were assigned by comparison of naked and in vivo patterns. The boundaries of protected regions are approximate due to typical sequence preferences in the DNase I digestion. [Lanes 1 and 5] In vitro purified (naked) DNA not treated with DNase I; [lanes 3 and 7] DNAs purified from nuclei without the addition of exogenous DNase I; [lanes 2 and 6] in vitro purified [naked] DNA treated with DNase I in vitro, [lanes 4 and 8] DNAs form nuclei treated with DNase I in vivo. These data are summarized in Fig. 3.
SP1 were used to protect MT-I and SV40 promoter fragments from DNase I digestion (Fig. 5). The only sites protected in MT-I are the two identified above by in vivo footprinting and sequence similarity. The distal site is protected at lower SP1 concentrations than is the proximal site. The size of the region protected in both cases is 18–20 bases, in agreement with the in vivo DNase footprint and with other in vitro SP1 footprints [Gidoni et al. 1984; Jones and Tjian 1985; Jones et al. 1986]. In this experiment, the parallel SV40 footprint provides a convenient, well-characterized standard for SP1 binding. The distal murine MT-I site binds human SP1 with an affinity between that of strong and intermediate SV40 sites, whereas binding at the proximal MT site is similar to that at weak SV40 sites. In summary, all in vivo and in vitro data suggest that the sequence at −182 of the MT promoter is occupied by a murine SP1 protein in vivo. This is consistent with the fact that deletion analyses of MT-I indicate that sequence within this region contributes to both basal and induced expression (Carter et al. 1984; Searle et al. 1985). By contrast, the proximal SP1-B site at −139 presents a more complicated picture. Apparently it is a binding site for one or more proteins in vivo, and it can serve as an SP1 site in vitro, albeit a weak one. Whether SP1 is among the proteins that bind there in vivo remains uncertain (see below).

Some genetic analyses suggest that sequences which influence basal expression also amplify metal induction (Carter et al. 1984; Searle et al. 1985; Karin et al. 1984; Karin and Holtgreve 1984; Stuart et al. 1984). Is the SP1-like in vivo footprint in Figure 2 affected, either qualitatively or quantitatively, by metal induction? The data in Figures 2 and 3 show clearly that there is no major change in the footprint at the SP1-A site upon zinc induction. This is best visualized by inspection of the enlarged footprint in the inset (Figure 2b, top), and is illustrated in the densitometric scans and difference plots (Figure 2b, bottom); and is summarized in Figure 3. The difference plots of noninduced and naked DNA or induced and naked DNA pairs show clearly the SP1 footprint, and the difference plot between induced and noninduced samples reveals the striking similarity of the SP1-A footprint in cells under both conditions. The same is true for cadmium induction [data not shown]. We conclude that if SP1 binding at the SP1-A site influences the level of expression during metal induction, it does not do so by simply increasing occupancy nor by a steric change that is strongly DMS sensitive. Metal induction does have a detectable effect on the DMS sensitivity of some guanines in the second potential SP1-B site located at −139, but the juxtaposition of this site with possible binding sites for other proteins may be responsible [see Discussion].

MLTF binds the murine MT-I gene in vivo

Another prominent set of hypersensitive and protected guanine residues comprise a clear in vivo DMS footprint between positions −94 and −105 [Figs. 2a and 3]. This region is also protected from DNase I digestion in nuclei of noninduced cells [Fig. 4]. Inspection of the sequence in this region revealed a 9 of 12 bp match to the recognition site of the major late transcription factor [MLTF], a cellular factor that was first identified by its activity at the major late promoter of adenovirus 2 [Ad 2] [Carthew et al. 1985; Miyamoto et al. 1985; Sawadogo and Roeder 1985; Chodosh et al. 1986]. Comparison of our in vivo DMS footprint with the in vitro DMS footprint for purified MLTF at the Ad2 promoter [Miyamoto et al. 1985]
shows that the patterns of protection from methylation are very similar [Fig. 6]. Moreover, recent in vitro footprinting experiments show that purified MLTF binds at this site in the murine MT-I promoter (Cathew et al. 1987). This region also contains a weaker sequence similarity (5 of 7) to the recognition site of transcription factor AP1, which has been shown to function in the human MT-IIA gene [Lee et al. 1987a,b]. AP1 activity can be modulated by the phorbol ester TPA [Angel et al. 1987], and genes containing an AP1 element can be induced by treating cells with TPA [Angel et al. 1986, 1987; Imbra and Karin 1987]. In an initial experiment, we tested whether expression of the MT-I genes in our A-60 cells is elevated in response to TPA. The data in Figure 1b show that the steady-state level of RNA was not affected by TPA treatment. This experiment is not decisive, but when the result is considered together with divergence from the consensus site, it suggests that AP1 is not responsible for the footprint centered around -100. We conclude that murine MLTF is bound to the MT-I gene in vivo at the site between -93 and -104. Our data also show that there is little change in occupancy at this site upon metal induction [Fig. 2a and summarized in Figs. 3 and 6].

Metal dependent protection of MRE sequences

All MT genes contain multiple copies of MREs [Carter et al. 1984; Karin et al. 1984; Stuart et al. 1984, 1985; Anderson et al. 1986; Maroni et al. 1986]. When two or more MREs are inserted upstream from a basal promoter, they can confer metal responsiveness upon that promoter [Searle et al. 1985; Stuart et al. 1985]. MREs are a family of moderately similar sequence of about 15 bp that are related to a rather loosely defined consensus sequence: CTNTCC/A/G/CNCGGCC, in which the underscored bases comprise the core consensus element. The five MREs of the mouse MT-I gene have been designated MRE-A through MRE-E, with MRE-A being closest to the start of transcription and MRE-E most distant [Stuart et al. 1984].

In noninduced cells, MREs A–E display a DNase I pattern that is essentially identical with naked DNA [Fig. 4], and they show very little protection from in vivo DMS treatment [Figs. 2, 3, 7], with the exception of MRE-D, which is discussed below. Upon stimulation with zinc, all of the MREs show protection of most guanines in vivo. The degree of protection ranges from 20% to 65% and corresponds well with the ability of each individual MRE to confer metal response upon a heterologous promoter [Stuart et al. 1985]. For example, MRE-E is unable to confer metal inducibility on the HSV-TK promoter, and it shows very little DMS protection here. The converse is true for elements A and C, which are more highly protected and are also potent metal response elements in synthetic promoters. Detectable protection from DMS is dependent on zinc induction at all sites except MRE-D, where metal treatment enhances a preexisting low-level protection. Slight hypersensitivity at the last three or four positions of most MREs is also observed. Figure 7 contains a summary of DMS footprints at MREs together with representative densitometry. A simple interpretation of the footprint pattern is that in the noninduced state MREs are not occupied, but upon metal induction they are protected due to the binding of positive-acting metal-responsive factors [MRF]. This agrees with data from in vivo competition studies which show that the limiting factors in metal induction act in a positive fashion [Seguin et al. 1984; Scholer et al. 1986; Mueller et al., in prep.). While this is the simplest view consistent with all data, the absence of a footprint under noninducing conditions must be interpreted cautiously, because it is expected that some interactions will escape detection, especially if they are relatively unstable or transitory.

Virtually all metal-dependent DMS protections can be accounted for by MREs A–E, with the noteworthy exception of a cluster of protected residues centered around –87 [Figs. 2 and 3]. This region contains a sequence that is moderately related to the MRE consensus: It matches at 9 of 15 positions overall, but possesses greater similarity in the MRE ‘core’, in which 4 of 5 bases agree. The DMS pattern is also quite similar to that at the other MREs [Fig. 7]. Some deletion analyses of this gene are consistent with the sequence functioning as a hitherto unidentified MRE [Searle et al. 1985]. Although these mutation data are not decisive, the sequence similarity, footprint pattern, and deletion data lead us to identify it provisionally as MRE-F. One potentially interesting distinction between MRE-F and the other MREs is that it appears to be less efficiently protected from DMS when cells are treated with cadmium than when they are induced with zinc [data not shown], whereas the others are affected identically by both inducers.
Footprinting amplified and transfected genes

In these experiments we have shown that in vivo footprinting studies can be executed successfully on transfected, amplified genes, and this approach should be generally applicable to promoters other than MT. It requires \( \geq 15 \) functional copies of the promoter per mammalian genome (S. Salser, unpubl.), although single-copy genes should be readily accessible in smaller genomes. In this study, cell lines containing sets of active genes were de-

Figure 7. Summary of in vivo DMS footprint patterns over MREs. The five previously identified MREs A-E (Stuart et al. 1984) are shown along with the potential MRE-F. All MREs are oriented with the coding strand on top as they appear in the promoter except MRE-B, which is inverted here to allow comparisons of the footprint patterns. Quantitation of DMS footprints under noninduced and Zn-induced conditions was performed as described in Materials and methods and is indicated by downward pointing arrows [↓] for protection, and by upward pointing arrows [↑] for hypersensitivities. Scale is at bottom of figure. An example of MRE densitometry is shown for the coding strand of MRE-B on the right half of the figure. The initial, normalized plots are superimposed pairwise as indicated and the corresponding difference plots are shown on the right. Beneath the densitometry plots is the coding strand sequence of MRE-B. [NA] In vitro purified DNA (naked); [−ZN] in vivo DNA, noninduced; [+ZN] in vivo DNA, Zn-induced.
rived by amplification of a chimeric MT–DHFR construct, but multiple-copy transformants could be equally useful. As in other transfection studies, it is necessary to select for footprinting transformants that show proper expression. Our cell lines were checked to ensure metal induction [Fig. 1b], and basal-level expression characteristic of the native MT gene was maintained by continuous selection for DHFR expression [see Materials and methods]. From the 70% and 65% protection at SP1 and MRE sites, respectively, it is clear that a large fraction of the transfected MT promoters in these cells is accessible to binding by cellular factors. It is worth noting that transfect target genes sometimes present specific limitations. For example, a peculiarity of mouse MT-I is that the endogenous gene is inducible by glucocorticoids, but no cloned DNA segment that retains this property upon transfection into cultured cells or into transgenic mice has been identified [Mayo et al. 1982; Pavlakis and Hamer 1983; Palmiter et al. 1982]. In agreement with these observations, we found that the MT-DHFR genes are not induced by dexamethasone, and therefore we did not attempt to footprint following hormone treatment.

Although in vivo footprinting is an effective method for visualizing a subset of protein : DNA interactions on a given gene, it is not expected that all protein : DNA contacts will be detected by this assay. For example, we do not observe significant footprinting over or near the TATA box [Figs. 2 and 3], even though these genes are being transcribed at a physiologically significant rate. This is also the case in several other in vivo footprinting studies of actively transcribing genes [Zinn and Maniatis 1983; Kemper et al. 1987]. Nevertheless, it is clear that a set of proteins in nuclear extracts do bind in vitro in other TATA containing genes [Davison et al. 1983; Parker and Topol 1984; Sawadogo and Roeder 1985], and previous genetic experiments have shown these sequences to be important for efficient MT-I transcription [Carter et al. 1984]. Perhaps the interactions that are observed in vivo at upstream elements are significantly more stable than are those that occur at the TATA sequence. In spite of these constraints, it is clear that we have successfully identified interactions at MRF, SP1, and MLTF binding sites in the murine MT-I promoter, and genetic analyses [Carter et al. 1984, Stuart et al. 1984, Searle et al. 1985] support the functional significance of these interactions. Based on these results, we suggest that it should now be efficient to survey other complex control regions in this way at an early stage in their characterization to focus subsequent mutagenesis or in vitro protein binding studies.

Figure 8. Evolutionary conservation of G elements in higher eukaryotic metallothioneins. Shown are the 22 G elements found in 11 MT promoters, and the consensus we have derived. General conservation of G rich regions has been noted previously [Karin 1984] and designated variously as a basal level enhancer [BLE] [Scholer et al. 1986], a part of the BLE [Karin et al. 1987], or a G-rich sequence [Seguin et al. 1986]. Numbers on either side of the G elements indicate their location in the promoters relative to the transcriptional start site. Arrows to the left of the sequences indicate the orientation of the elements in their promoter. All of the elements have been displayed in the orientation of the consensus for comparative purposes. The consensus along with the percent base usage is indicated at the bottom of the figure. Sources: Drosophila MT [Maroni et al. 1986]; human MT-Ia [Richards et al. 1984]; human MT-Ib [Heguy et al. 1986]; human MT-Ie [Schmidt et al. 1985]; human MT-II and human MT-Ig [Varshney et al. 1986]; human MT-II [Karin et al. 1987]; mouse MT-I [S.J.S. unpubl., see Fig. 3 legend and Glanville et al. 1981]; mouse MT-II [Searle et al. 1984]; rat MT-I [Andersen et al. 1986]; rat MT-II [R.D. Andersen, unpubl. 1986, NIH data base].
Discussion

We have used in vivo genomic footprinting with DMS and DNase I to observe DNA : protein interactions at the MT-I promoter (Figs. 3 and 7). These experiments led to definition of two sets of probable protein : DNA interactions as they occur in cells under noninducing and inducing conditions. The results obtained allowed us to identify a new metal-responsive element, to limit the possible mechanisms for interaction between basal-level elements and metal-responsive elements, and to begin to resolve how overlapping, and thus potentially competing, recognition sites are actually used.

It is interesting to compare the pattern of in vivo protection at MREs with in vitro studies of Seguin and Hamer (1987). They observed a cadmium-dependent Exo III footprint at MRE-D when MT-I DNA was incubated with a nuclear extract. Our results differ from their in vitro data in two respects. First, they detect binding only in the presence of cadmium but not in the presence of zinc, whereas we find similar in vivo footprints with both metals at MREs A-E. Second, they observe binding at just one MRE, whereas we see footprints at MREs A-E and F. The general pattern of in vivo interactions we have observed is, however, in close agreement with in vivo studies reported recently by Herschman and colleagues (Anderson et al. 1987) for the related rat MT-I gene. We do not know the basis for the differences between in vivo and in vitro results, although it is interesting that our data find MRE-D to be the only metal response element showing a detectable footprint in the absence of added metal (Fig. 7). It may also be relevant that MRE-D (−150 to −136) overlaps the potential binding site SP1-B (−143 to −134), as discussed below.

Overlapping recognition sites and possible competition among factors

The presence of overlapping potential binding sites at the MRE-D/SP1-B region raises the possibility of competition among several different factors. DNA sequence data alone do not provide enough information to predict which factors, if any, will be bound at this site in the cell. The in vitro SP1 footprint data (Fig. 5) establish that this sequence can serve as a SP1 binding site in the absence of competing factors, which leaves open the possibility that it may function as a SP1 site in vivo. The in vivo DNase (Fig. 4) and DMS (Fig. 2) footprints show that this region is occupied by factors in L cells, but the absence of the characteristic SP1 DMS footprint pattern makes it unlikely that SP1 is responsible. Another possibility is that, in both the induced and noninduced state, there is significant binding of metal-responsive factors at this site. This alternative is supported by the pattern of DMS footprinting. The DMS pattern at MRE-D in noninduced cells is qualitatively similar to that at other functional MREs after metal induction. The induced pattern at MRE-D is quantitatively increased upon metal induction but not qualitatively changed from its basal pattern. It is interesting that MRE-D is the most effective MRE when present in multiple copies in a synthetic promoter (Stuart et al. 1985) and is also the only MRE to show metal-dependent protein binding in vitro (Seguin and Hamer 1987).

For the case of the MRE-D/SP1-B region, our working model is that in L cells the balance between active SP1 and MRFs favors binding by MRF, but this may not be the case in other cell types or under different physiological conditions. The notion of in vivo competition for overlapping sites has some appeal because it provides a potentially sensitive mechanism for regulating gene expression in different cell types or tissues in response to modest differences in relative levels of participating factors. Small genetic changes can also tip the balance between two binding sites, and such changes may be important in the evolution of different patterns of expression. An inadvertent experimental example was provided when Stuart et al. (1985) placed oligomers containing the MRE-D overlap region in front of a truncated HSV-TK promoter containing only a TATA element. The wild-type MRE-D region made TK expression metal inducible. Surprisingly, they found that if the oligomer was modified so as to make it a better match to the SP1 consensus without disrupting the MRE, the gene was no longer inducible by metals, but exhibited a 10-fold elevation in basal expression. Competition among factors for binding in this region may also contribute to the multiplicity of protein : DNA complexes observed as distinct electrophoretic species when DNA is incubated with crude nuclear extracts (Seguin and Hamer 1987).

The phenomenon of overlapping, potentially competing recognition sites is probably not confined to the MRE-D/SP1-B region. All higher eukaryotic MT genes for which sequence data are available contain one or more copies of a conserved 14-bp G-rich sequence, and from 22 examples we have derived a consensus element G(C/G)GGG(C/G)CG(T/G)GTGCA (Fig. 8). A function has not yet been defined for this sequence feature, so it is simply referred to here as the G element or G box. Murine MT-I contains two of these elements: one centered around −140, the other centered around −100. Like other MT G-boxes, these overlap other potential recognition sites. The site centered at −140 overlaps the MRE-D and SP1-B sites, and the element centered at −100 overlaps the MLTF site. In other MT genes (for example, human MT-IIA, human MT-IA, mouse MT-II, and Drosophila MT) G elements overlap potential SP1 and MRE sites. Our footprinting data did not detect interactions specifically attributable to G elements, and existing mutational studies assayed in various cell lines have also failed to detect a specific G-box function. On the other hand, the presence of G-elements in all MT genes, together with their substantial sequence similarity, suggest that they are important enough to demand evolutionary conservation, probably because they serve as recognition sites for one or more factors. It seems possible that the G elements may be required in a physiological context that has not been tested by the transfection assays and footprinting studies.
**Interaction of MREs and basal elements**

Although multiple MREs are sufficient to direct metal-inducible transcription from heterologous promoters, the absolute level of expression depends on additional non-MRE upstream elements. For example, Stuart et al. (1985) found that in the context of a synthetic promoter, SP1 elements amplify the MRE-mediated response, and promoter mutations generally support this view. The effect is not simply additive, but is synergistic. A survey of different MT regulatory regions shows that the theme of multiple MREs combined with several potential basal elements is common, but there is considerable flexibility in basal element identity and sequence organization. This suggests that the capacity of basal element factors to enhance MRE activity is a general property shared by several factors including SP1 (mouse MT-I, human MT-IIA), AP1 (human MT-IIA), and MLTF (mouse MT-I).

How do basal elements amplify metal induction? The constitutive occupancy of SP1 and MLTF sites in vivo rules out one straightforward mechanism for amplifying metal-dependent expression: Active MREs do not function by simply increasing the efficiency of factor binding at basal element sites. The possibilies that remain fall into two classes. MREs and basal elements both may act by increasing directly the rate of initiation. Alternatively, basal elements might alter the rate of metal-induced transcription by increasing the accessibility of DNA to factors that do govern initiation—possibly MREs. These different functions for basal elements are not easily distinguished by standard cell transfection assays or by current in vitro transcription systems, although it should now be possible to introduce appropriately mutated promoters and observe how ablation of one or more of the basal elements affects the in vivo protein:DNA interactions at MREs.

**Materials and methods**

**Plasmid construction**

Plasmid MD was constructed from the mouse MT-I genomic sequences of pEE-3.8 (a gift from R. Palmiter) and the DHFR sequences of SV3-DHFR (Southern and Berg 1982). The MT-I promoter fragment used in this plasmid contains ~1775 bp of 5' untranslated sequences between EcoRI and BglII. The BgIII site ends 8 bp on the 5' side of the MT translation start. The 3' MT fragment contains the complete second intron and poly[A] addition site of MT-I as an *AatI-*HindIII fragment, with the *AatI* site converted to a *BglII* site. These fragments were inserted into pBR322 to create pMT-33. The coding region of DHFR, along with 60 bp on the 5' side of and 80 bp on the 3' side of the translation start and stop respectively, was removed from SV3-DHFR as a *FnuDII* fragment and inserted into the *BglII* site of pMT-33 after addition of a *BamHI* linker at the *FnuDII* site.

**Cell culture**

Murine cell lines A-0.3 (formerly KTO-A, Kim and Wold 1985) and A-60 were gifts from S. Kim, and were adapted for growth in dialyzed calf serum. These lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed calf serum (Gibco), 100 U/ml penicillin G, 7.5 U/ml streptomycin, 4 μg/ml azaserine, 15 μg/ml adenosine, and 3 x nonessential amino acids. Dialysis of the serum increased the induction ratio of MT genes ~twofold by lowering the basal level of expression [P.R. Mueller, unpubl.]. A-0.3 and A-60 were further supplemented with 0.3 μM and 60 μM methotrexate (MTX), respectively. The presence of MTX did not influence the expression or induction of the MT genes (data not shown). A-0.3 and A-60 contain 100 and 700 copies of pMD, respectively [J. Jong, unpubl.].

**RNA preparation and measurement**

Cells were ~70% confluent before induction. Four or 8 hr prior to harvesting the RNA, fresh media were added to the plates with or without 130 μM zinc sulfate. Prior to TPA (12-O-tetradecanoyl-phorbol-13-acetate) inductions, cells were washed with PBS and transferred to media containing 0.5% dialyzed calf serum, without azaserine or methotrexate (MTX) for 24 hr. TPA (in ETOH) or an equivalent volume of ETOH [14.4 μl] was then added to the serum-starved media for an additional 12 hr. Final concentration of TPA was 60 ng/ml. Total RNA for each condition was prepared from two 150-cm plates according to the method of Chirgwin et al. (1979), as modified by Ngai et al. (1984). RNA was further purified by extraction with phenol/Sevag and precipitation with isopropanol and then ethanol. Subsequent treatment with RNase-free DNase had no effect on RNA quantitation (data not shown), so this was not done routinely.

RNase protection was performed as described by Zinn et al. (1983) and modified by Kim and Wold (1985). Fifteen micrograms of total RNA was hybridized with a molar excess (12 ng, or 280 fmoles) of labeled probe of specific activity 7 × 10⁵ cpm/μg. The probe used protects the DHFR sequences in the construct and the endogenous DHFR, but the endogenous signal is much weaker than the strong construct signal and cannot be seen on short exposures. Longer exposures of RNA experiments performed on the parental cell lines show that the protected endogenous band is ~15 bases shorter than the protected construct band (P. Mueller, unpubl.). This is consistent with the major transcriptional start site of mouse DHFR (Farnham and Schimke 1986). Gel slices containing the protected probe were excised and counted for quantitation.

**In vivo and in vitro DMS/piperidine cleavage of DNA**

Cells for in vivo footprinting were treated identically to those used for RNA measurements. After 4 hr with or without 130 μM zinc sulfate, the media was replaced with media containing 1 μl/ml DMS (DMS was added immediately before use). This replacement media also contained 130 mM zinc sulfate, if appropriate, and was prewarmed to 37°C in a 4% CO₂ environment. The DMS was allowed to react with the cells for 2 min, at which time the DMS-containing media was removed and the plates immediately were rinsed once with 37°C PBSA and subsequently washed 3 x with 37°C PBSA for 30 sec with gentle shaking. Cells were lysed and scraped from each plate in 1.5 ml of DNA harvest buffer [1 mM Tris (pH 7.5), 400 mM NaCl, 2 mM EDTA, 0.2% SDS, 0.2 mg/ml protease K], and this mixture was incubated with occasional mixing for an additional 3–5 hr.
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at 37°C. DNA was extracted once each with phenol, phenol/
Sevag, and Sevag; and twice with ether. The DNA was then precipitated once with isopropanol and once with ethanol before resuspending it in 10 mM Tris (pH 7.5), 1 mM EDTA. The final nucleic acid concentration was 1–1.5 mg/ml, of which 50–60% was DNA. To precipitate the in vivo methylated DNA in parallel with the in vitro methylated DNA, 200 μl of this mixture was mixed with 50 μl of 1.5 M sodium acetate (pH 7.0), 1 M β-mercaptoethanol, 100 μg/ml yeast tRNA, and 750 μl of ethanol. The pelleted DNA was washed once with 75% ethanol before being suspended in 200 μl of 1 M piperidine, and then heated to 90°C for 30 min. Piperidine treatment cleaves DNA at methylated guanines and eliminates contaminating RNA by base hydrolysis. Piperidine was removed by vacuum followed by two cycles of washing and precipitation.

DNA for in vitro DMS treatment (naked) was prepared identically, except the in vivo DMS treatment step was omitted. This DNA was methylated in vitro by adding 0.9 μl of DMS to and then heated to 90°C for 30 min. Piperidine treatment cleaves DNA at methylated guanines and eliminates contaminating RNA by base hydrolysis. Piperidine was removed by vacuum followed by two cycles of washing and precipitation.

DNA for in vitro DMS treatment (naked) was prepared identically, except the in vivo DMS treatment step was omitted. This DNA was methylated in vitro by adding 0.9 μl of DMS to 200 μl of DNA (~1 mg/ml) for 30 sec at room temperature. This DMS condition was empirically determined to match the in vivo DMS conditions. Under methylation would show a general underrepresentation of lower-molecular-weight bands and overrepresentation of high-molecular-weight bands. The converse is true for overmethylation. Such mismatches in methylation conditions do not, however, produce specific local patterns of protection or hypersensitivity that would be easily mistaken for protein:DNA footprints. The reaction was stopped with 50 μl ice-cold stop buffer [1.5 mM sodium acetate (pH 7.0), 1 M β-mercaptoethanol, 100 μg/ml yeast tRNA] and 750 μl of ethanol chilled to −70°C. The samples were then piperidine treated in parallel with the in vivo samples. For in vitro controls that were not reacted with DMS or piperidine, the DNA was first cleaved with EcoRI and RNase A to reduce viscosity and remove contaminating RNA, respectively, and then processed in parallel with the other samples.

In vivo and in vitro genomic DNase treatment

Cells for in vivo DNase I footprinting were treated identically to those used for RNA measurements. Preparation of nuclei and in vivo DNase treatment was performed as described by Zinn and Maniatis (1985) with slight modifications. Naked DNA [in vitro] was prepared by the conditions described above, and digested with DNase I [Boehringer Mannheim Biochemicals] to the same extent as the nuclear samples [empirically determined]. Underdigestion would show a general underrepresentation of lower-molecular-weight bands and overrepresentation of high-molecular-weight bands. The converse is true for overdigestion. Such mismatches in DNase I conditions do not, however, produce specific local patterns of protection that would be easily mistaken for protein:DNA footprints. Both the in vitro and the in vivo DNAs were also digested with EcoRI and RNase A to reduce viscosity and remove contaminating RNA.

Primer extension of DMS and DNase-cleaved genomic DNA

Visualizing the footprint patterns by primer extension eliminates the technically challenging blotting of sequencing gels and subsequent hybridization (Church and Gilbert 1984). In addition, extension of an end-labeled primer ensures that single-strand resolution is not compromised by either radioactive decay of a multiply end-labeled probe or imprecise action of a single-stranded nuclease [Jackson and Felsenfeld 1985]. Because loading more than ~75 μg of DNA per lane (lane size 8 mm x 0.8 mm) leads to decreased resolution, our procedure required ~15 copies per mammalian genome [P. Mueller, unpub.]. Specifically, 65 μg A-0.3 DNA or 14 μg of A-60 DNA were mixed with 0.18 pmole labeled primer [sp. act. 6–14 × 10⁷/pmol] and suspended in 55% deionized formamide, 6 × SSPE [900 mM NaCl, 60 mM NaH₂PO₄, H₂O], 6 mM EDTA [pH 7.4], final volume was 70 μl. The primers used were complementary to −227 to −203 on the noncoding strand and +47 to +23 on the coding strand. These 25-mers have a GC content of 60% and 52%, respectively. Samples were heated to 95°C for 5 min and incubated for 30–60 min at 30°C. These conditions proved to maximize the amount of primer hybridized while minimizing the self-hybridization of the genomic DNA and nonspecific background hybridization of the primers [P. Mueller, unpub.]. Different length or GC content of the primers might require different hybridization conditions. To remove excess primer and change the reaction buffer, one of the two methods was used. For the DNase and early DMS experiments, the hybridization mixture was run over a quick-spin column of Sephadex G-50 equilibrated in low KT buffer [12.5 mM KCl, 12.5 mM Tris, pH 8.3 at 50°C], and the volume of the efflux was adjusted to 87.5 μl with low KT buffer. In other experiments, the hybridization mixture was diluted to a volume of 670 μl with 2.25 mM ammonium acetate and then precipitated with isopropanol. The pelleted DNA was then dissolved in 87.5 μl of low KT buffer. In either case the DNA was then mixed with 12.5 μl 8 × AMV reverse transcription buffer so that the final concentrations were 50 mM KCl, 50 mM Tris, pH 8.3 at 50°C, 8 mM MgCl₂, 0.8 mM dNTP, and 4 mM DTT. Two micro-liters [28 units] AMV reverse transcriptase [Life Sciences, Inc.] were added and primers were extended for 30 min at 50°C. The reaction was terminated by adding 3 μl 500 mM EDTA, 35 μl 8 mM ammonium acetate, and 10–20 μg yeast tRNA. Samples were precipitated with 1 volume isopropanol, suspended in 12 μl loading buffer [80% deionized formamide, 40 mM Tris-borate [pH 7.5]] and run on 8% denaturing polyacrylamide gels 0.8-mm thick. Gels were dried and exposed for 1–10 days without an intensifying screen.

In vitro footprinting of SP1

In vitro DNase I footprints were performed, as described by Briggs et al. [1986], with 95% homogeneous, human SP1 that was ~10 ng/ml [a gift from J. Kadonaga and R. Tjian]. The mouse 400-bp MT-1 promoter fragment was labeled at the BamHI site of plasmid MT-1ai [a gift from R. Palmiter], and the SV40 326 bp promoter fragment was labeled at the HindIII site of plasmid SV2-DHFR [Southern and Berg 1982], 6.5 fmoles of each of these end-labeled DNA fragments were used and 8 ng/ml was the final DNase I concentration [Boehringer Mannheim Biochemicals]. Gels were run as above.

Quantitation of DMS footprints

Several exposures of footprinting gels were prepared using Kodak XAR-5 film without an intensifying screen. Densitometry was performed with a LKB Ultrascan XL laser densitometer, which has a beam size of 800 μm by 50 μm. Each lane was scanned nine times with a 200-μm overlap between scans and a 120-μm vertical step. These scans were analyzed using the Turbo-Densitometry program [S.I.S.]. Averaging of the nine scans produced a single composite scan per lane. To compare the various lanes of each loading [i.e., naked, in vivo noninduced, and in vivo induced], the composite scans were aligned, and the signal normalized. This normalization was to sequences in the guanine ladder that lacked both binding sites and observable footprints; this compensated for minor variations in sample loading, and had only a slight effect (<10%) on the final quantitation of protection and hypersensitivity. To
quantitate individual protections and hypersensitivities, the integrated area for each base in the original plots was subtracted from the corresponding area in the naked DNA plot, and the difference was divided by the area of the naked DNA base. Difference plots are useful for illustrating footprints and comparing occupancy under noninduced and induced conditions. These were obtained by subtracting the digitized and normalized scan of one lane from that of another lane on a point-by-point basis. These plots indicate regions of protection above the baseline and regions of hypersensitivity below the baseline.

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