Zinc dependent binding of a liver nuclear factor to metal response element MRE-a of the mouse metallothionein-I gene and variant sequences

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ABSTRACT

Metallothionein gene transcription is inducible by zinc and other heavy metals, and several metal response elements (MREs) have been mapped within about 200 bp upstream of the site of transcription initiation in several metallothionein genes. Comparison of a number of MREs defined a 15 bp consensus sequence containing a more highly conserved MRE core sequence TGCRCNCG. I have used the proximal MRE of the mouse metallothionein-I gene (MRE-a) in DNA fragment mobility shift assays to detect a protein in rat liver nuclear extracts which binds specifically to the MRE in a zinc-regulated manner. Use of a comprehensive series of variant MRE sequences established that the binding was strongly dependent on the MRE core sequence, whereas changes at the less highly conserved positions had minor effects on binding. This provides strong evidence that the protein detected is responsible for the zinc-responsiveness of the MT genes in liver, and provides a more detailed picture of the regulatory protein:MRE interaction than was previously available.

INTRODUCTION

Zinc is an essential trace element with both structural and catalytic functions in a variety of enzymes (1). Zinc was also found to be required by the 5S RNA gene transcription factor TFIIIA for binding to DNA (2), and it was suggested that the zinc ions stabilize the folding of a repeated 30 amino acid DNA binding domain by coordination of characteristically positioned cysteine and histidine residues (3). This structural feature was named the zinc finger. Similar zinc-finger motifs have subsequently been recognised in many other DNA-binding proteins (4), including the mammalian transcription factor Sp1 which activates transcription from a variety of viral and cellular promoters (5). Zinc is essential for the DNA-binding activity of these transcription factors. It appears that the zinc plays an essentially structural, rather than regulatory function because the proteins normally contain bound zinc in the cell (4,5,6).

Homeostasis of the zinc pool within the organism is believed to be a major function of the metallothioneins (7,8). These are small, cysteine-rich proteins with the ability to coordinate a number of ions of zinc, cadmium or copper. Metallothionein (MT) genes can be expressed in most tissues, consistent with a 'housekeeping' role, although levels may be particularly high in liver and kidney (7,8,9). Their expression can be induced above a low basal level by certain stress-related inducers (10,11,12), or by elevated concentrations of heavy metals (9,13); and the resulting increased levels of metallothionein can provide some protection against toxic effects of the heavy metals (13). Several groups analysing the promoter regions of MT genes have identified metal response elements (MREs) which mediate the transcriptional induction by heavy metals (14,15,16). For example, six MREs (named MRE-a to MRE-f) have been identified within 200 bp upstream of the mouse MT-I gene (15,16,17), although it appears only MRE-a, -b, -c and -d are sufficiently active to function independently (18).

Evidence that the MREs function by binding a positively acting transcription factor in the presence of heavy metals (i.e. function by activation rather than repression) was provided by competition experiments (19,20) and by analysis of model promoters with or without MREs (21). Footprinting experiments have detected metal-dependent binding of factors to MREs *in vivo* (17,22). It seems likely that the MRE- binding factor is a protein which requires bound zinc (or other heavy metal) ions in order to assume a conformation able to bind to DNA; it will be of interest to determine whether this protein contains 'zinc-finger'- like structures having a relatively weak affinity for zinc, such that this DNA-binding motif only folds correctly at elevated zinc concentrations, or whether there is some other mechanism. In the analogous yeast system (which however responds to copper but not zinc) the regulator *ACE1* of yeast copperthionein *CUP1* has recently been cloned and it was proposed that the DNA-binding N-terminal domain assumes a copper-cluster type of structure similar to that of metallothionein itself (23).

Factors have been detected in nuclear protein extracts from mouse or human cell lines which bind to MRE-d of the mouse MT-I promoter in a zinc- or cadmium- regulated manner (24,25,26). This MRE, at −150 to −136 bp, appears to be the strongest MRE in this promoter (18,24); however analysis of MRE-d is complicated by overlap with a sequence apparently able to bind the widely distributed transcriptional activator Sp1 (17,26).

Another protein has recently been purified from mouse L-cells on the basis of its affinity for a trout MRE (27). Curiously, this protein only gave a footprint on MRE-e of the mouse MT-I promoter (−175 to −161), and yet this MRE was shown to have minimal regulatory activity as detected either by analysis of 5′

deletions (15,16), or when tested in isolation (21). This raises the interesting possibility that there might be a family of transcription factors specific for different MRE sequence variants, which could provide the basis for differential regulation of MT genes by different heavy metals and in different tissues (28,29).

MRE-a of the mouse MT-I gene has been shown capable of independent regulatory function (18,21), which is not complicated by overlap with binding sites for Sp1. This paper describes the detection in rat liver nuclear extracts of a protein which binds to mouse MRE-a in a zinc- dependent manner, and examines its binding to a series of MRE variants differing from the optimised recognition sequence CTTTGCACCCGGACT by single nucleotide substitutions. The good correlation between its target site specificity and the previously defined MRE core sequence suggest this protein may play a role in the induction of MT gene transcription in the liver in response to zinc.

MATERIALS AND METHODS

Nuclear extracts

Nuclear extracts were prepared from liver of Hooded Lister rats essentially according to the method of Schibler and coworkers (30,31).

Oligonucleotides and MRE probes

MRE oligonucleotides were a kind gift from J. Habener and R. Palmiter, or were obtained from Alta Bioscience, Birmingham, UK. All MRE oligonucleotides were synthesized as self-complementary 38-mers with BamH1-site compatible ends, following the pattern 5'-GATCCAGTCCGGGCGCAAAGGC-CTTTGCGCCCGGACTG-3' for MRE-a; the two MRE-a sequences in opposite orientations are shown underlined. Variant MRE oligonucleotides were altered in both copies of the MRE sequence to maintain self-complementarity. Oligonucleotides were used directly for the competition experiment. However, each oligonucleotide was also cloned into the BamHI site of a pBR322-derived plasmid pMK5'Δ-42, and checked by DNA sequencing. These cloned versions of the variant MREs were used for the generation of radioactive MRE monomer probes for gel retardation assays.

MRE monomers were released from the plasmid vectors by digestion with BamHI (which cut at either end of the cloned oligonucleotides) and either Stu I or Hae III, which cut in the centre between the two divergent MREs. The Stu I site was destroyed in C1 variants; therefore Mlu I was used for C1→G, and Hind III for C1→T. C1→A was excised with BamH1 alone to give a dimer, and hairpin-ended monomers were generated by boiling and rapid cooling. The DNA fragments were radioactively labelled by endfilling using Klenow fragment and α^{32}P-dATP or dCTP, the other deoxynucleotides being present unlabelled. Labelled MRE monomers were purified by electrophoresis through a 12% polyacrylamide gel, visualized by autoradiography, excised, and eluted by shaking the pulverised gel pieces overnight at 37° in 0.25−1 ml 10 mM Tris pH8.

DNA fragment mobility shift assays

The basic methodology of detection of site-specific DNA binding proteins by their ability to retard the migration of DNA fragments during electrophoresis in nondenaturing polyacrylamide gels was described previously (32,33).

Binding reactions

Binding was performed in 20 μl of 25mM HEPES pH7.9, 12% glycerol, 10 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.6mM DTT. Reactions also contained either $ZnSO_4$ at 60−100 mM or as otherwise stated; or EDTA at 0.1 mM; or 1,10-phenanthroline (a more efficient chelator of zinc) at 0.5mM. Poly(dIdC).poly(dIdC) (Pharmacia) was added as a non-specific DNA competitor, generally at 1 μg per reaction unless otherwise stated. Each sample received 10−15 fmol of the appropriate MRE probe, containing approximately 10,000 cpm (Cerenkov). The protein extract was added last, 4−5 μg in a volume of 1 μl. Binding was performed for 20−30 minutes at room temperature unless otherwise indicated, then the samples were loaded and electrophoresed at 12−15 V/cm on 5% polyacrylamide gels (30:0.8 acrylamide:bisacrylamide). Gels were prepared in 22 mM Tris, 22 mM borate, and were pre-electrophoresed for 30 minutes prior to loading the samples. Gels were dried and autoradiographed by standard procedures. Densitometry for Figure 3 was performed on multiple exposures of each gel using an LKB Ultrascan XL densitometer.

RESULTS

Detection of a factor binding to MRE-a

The relative positions of the metal response elements within the promoter region of the mouse metallothionein-I (MT-I) gene are indicated in the upper part of Figure 1. Elements a, b, c and d have been shown to be capable of acting independently in a model promoter when transfected into tissue culture cells, whereas MRE-e was inactive in this assay (18). The results of *in vivo* footprinting experiments agreed with the functional studies, showing greater zinc-induced protection of MREs -a, -b, -c and -d than of MRE-e (17). These studies also detected an additional zinc-dependent footprint, between bp −94 and −80 of the promoter; this site was named MRE-f. Binding sites for the transcription factors Sp1 and MLTF are also indicated

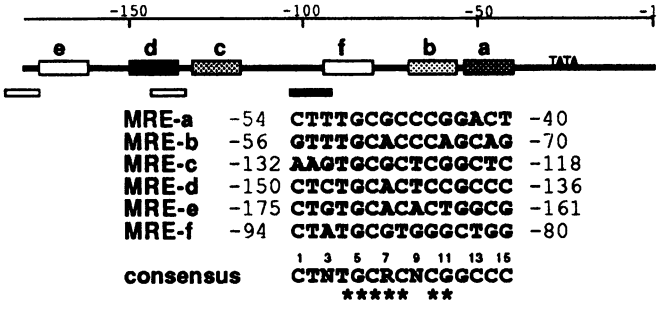

Figure 1. Organisation of the mouse metallothionein-I gene promoter region. DNA sequences from −1 to −180 bp upstream from the site of transcription initiation are represented by the thick black line. Metal response elements MRE-a to MRE-f are indicated as rectangles on the line; stronger function is indicated by darker shading. Binding sites for other transcription factors are shown below the line; open rectangles indicate the position of Sp1 binding sites and the black rectangle indicates a binding site for MLTF. The nucleotide sequences of the MREs are aligned in the lower part of the figure together with a consensus sequence derived by comparison of MREs from metallothionein genes from several species. The numbering indicated above the consensus sequence is used throughout this paper. The more highly conserved MRE core sequence is indicated by asterisks. R=purine; N=any nucleotide.

(17,34); these may both contribute to the basal level of MT gene transcription, and also cooperate with MREs in the response to heavy metals.

The sequences of the MREs are aligned in the lower part of Figure 1, together with a consensus sequence derived by comparison of MREs from several mamalian MT genes (18). The numbering of the basepairs shown above the consensus will be used to simplify disussion of all MREs in this paper. As reflected in the consensus sequence, the 3′ end of the highly conserved MRE core sequence TGCRCNCG (positions 4 to 11) frequently overlaps a sequence rich in C and G (positions 7 to 15), reminiscent of sequences bound by Sp1 (35). Of the MREs in the mouse MT-I gene this feature is most evident in MRE-d, and DNA fragment mobility shift assays have provided evidence for an Sp1-like activity binding to this sequence (26). Other MREs such as MRE-a do not share this extended C+G rich characteristic, and are therefore unlikely to bind Sp1.

Because MRE-a has been clearly shown to possess regulatory activity while being uncompromised by possible simultaneous binding of Sp1 to an adjacent or overlapping site, I initially used MRE-a to assay for zinc-dependent binding proteins present in a rat liver nuclear extract, using a DNA fragment electrophoretic mobility shift assay (32,33). Nuclear proteins were incubated with a radioactive 21 bp MRE-a probe together with a range of concentrations of the nonspecific competitor poly(dIdC), in the presence of either 100 μM EDTA or 60 μM zinc sulphate, prior to electrophoresis on a nondenaturing 5% polyacrylamide gel. As shown in Figure 2, at low competitor concentrations several prominent bands corresponding to retarded protein:MRE complexes were observed in both the absence and presence of zinc (lanes 1−4 and 9−12). However the intensity of one band (indicated ZDC, zinc dependent complex) was greatly increased in the presence of zinc, and persisted to higher levels of competitor (compare lanes 11−15 with lanes 3−7). The differential sensitivity of the bands to non-specific competition suggests that the ZDC is due to relatively tight, sequence-specific binding of a protein to the MRE-a probe whereas the other bands

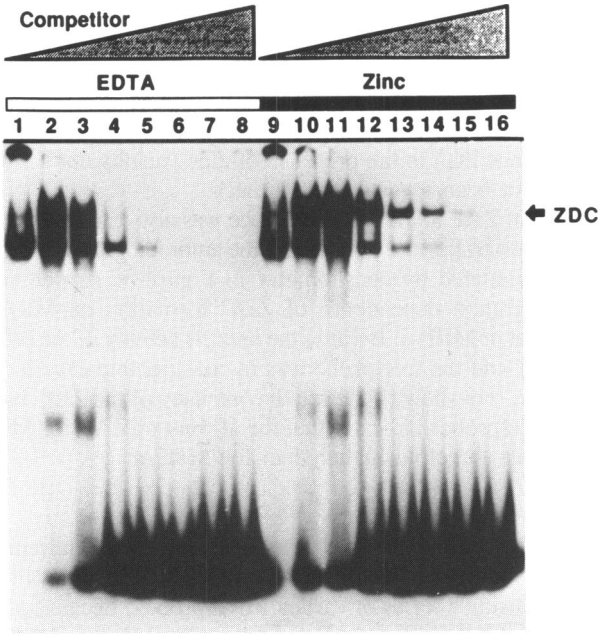

Figure 2. Gel retardation assays using MRE-a probe; titration of nonspecific competitor in presence of EDTA or zinc. Rat liver nuclear proteins (4 μg) were incubated with a ^{32}P-labelled 21 bp probe CCTTTGCGCCCGGACTGGATC containing the sequence of MRE-a (underlined), in the presence of 100 μM EDTA (lanes 1 to 8) or 60 μM $ZnSO_4$ (lanes 9 to 16). Each series of reactions was performed in the presence of a range of concentrations of nonspecific competitor DNA as follows: series 1 to 8 and 9 to 16 each contained respectively 0, 0.03, 0.06, 0.12, 0.25, 0.5, 1, 2 and 4 μg poly(dIdC). The major band at the bottom of the gel is the free probe; ZDC=zinc dependent complex.

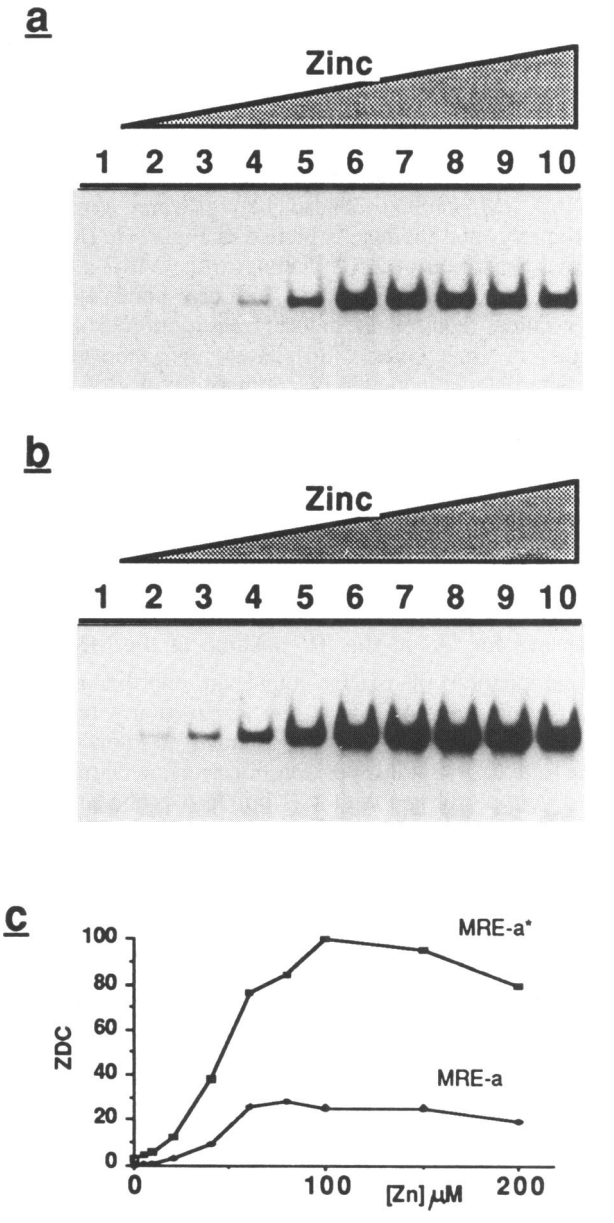

Figure 3. Dose response of zinc required for formation of zinc dependent complex. Nuclear proteins were incubated with 21 bp radioactive probes; a) MRE-a probe CCTTTGCGCCCGGACTGGATC b) MRE-a* probe CCTTTGCA-CCCGGACTGGATC (MREs underlined, differences shown in bold type). Binding reactions contained 0, 5, 10, 20, 40, 60, 80, 100, 150 or 200 μM $ZnSO_4$ in lanes 1 to 10 respectively. Only the region of the gel containing the zinc-dependent complex is shown; apart from the unbound probes there were no other major bands. c) Densitometric comparison of the bands shown in a) and b); lower line−MRE-a; upper line−MREa*.

which predominate at low levels of competitor are due to relatively weak, nonspecific binding by more abundant DNA binding proteins. Furthermore, whereas the bands observed at low concentrations of competitor were rather variable, the ZDC was reproducibly observed with several different protein preparations and under different experimental conditions. The protein in this complex is a likely candidate for the zinc-dependent regulator of MT gene transcription. The MRE-a binding protein detected by formation of the ZDC will be referred to as ZAP (zinc activated protein).

Dose-response of ZDC formation

To determine the concentration of zinc required for maximal ZDC formation, a range of concentrations between 0 and 200 μM was included in the binding reaction with the nuclear extract and MRE-a probe. As shown by the gel retardation assay (Figure 3a), the ZDC band reached a maximum intensity between 60 and 100 μM $ZnSO_4$. These bands were compared by densitometry, and the results plotted in Figure 3c (lower line). Maximal activation of ZAP binding to the MRE-a probe was obtained with 60–100 μM zinc. This corresponds well with the concentration required to induce transcription from the MT promoter in tissue culture (36). However, whereas MT gene expression is also inducible by cadmium (typically 5–20 μM) and copper (50–200 μM; 13,36), neither cadmium (1–60 μM) nor copper (5–200 μM) promoted the formation of this protein-DNA complex in the mobility shift assay, nor were any new bands observed (results not shown). It is unclear whether the protein involved in the ZDC is not activated by these metals, or whether other components of the binding reaction made the cadmium and copper unavailable for binding by ZAP.

Preference for 'A' at the 'R' position of the MRE core

The compilation of MREs found an approximately equal frequency of either A or G at the 'R' position within the core sequence TGCRCNCG (18). In contrast, no known functional MREs have C or T at this position. Electrophoretic mobility shift assays were performed with a 21 bp DNA probe similar to the MRE-a probe but incorporating the TGCACCCG version of the MRE core sequence. This altered MRE-a sequence has been named MRE-a*. Binding was performed in the presence of either EDTA or zinc, and with a range of concentrations of the nonspecific DNA competitor poly(dIdC) (as with MRE-a previously; Figure 2). The results are shown in Figure 4. A prominent zinc-dependent band was observed (compare lanes 12–16 with lanes 4–8), which was resistant to high concentrations of poly(dIdC). This complex comigrates with the ZDC formed on the MRE-a probe (Figure 2), suggesting that the same protein may be involved (this was confirmed by the competition assay described below, Figure 6). The MRE-a*:ZAP complex appears to be more stable than that containing the original MRE-a, as shown both by the presence of more ZDC at a given concentration of poly(dIdC), and greater resistance to higher levels of the nonspecific competitor. In contrast, substitution of C or T at the 'R' position greatly inhibited formation of the ZDC (unpublished results and Figure 5).

In addition to the nonspecific protein:DNA complexes observed at low concentrations of poly(dIdC) which were similar to those obtained with the MRE-a probe, an additional protein:DNA complex of greater mobility than the ZDC already discussed was also increased in the presence of zinc on the MRE-a* probe (Figure 4). This complex was more susceptible to nonspecific competition than the ZDC and also showed much greater variability between experiments, in some cases being stronger in the absence than in the presence of zinc (results not shown). This has not been investigated further.

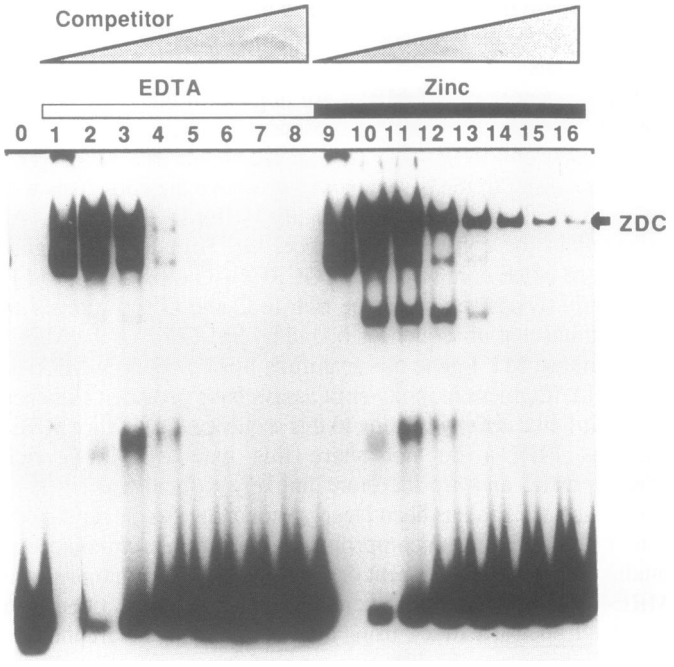

Figure 4. Gel retardation assays using MRE-a* probe; titration of nonspecific competitor in presence of EDTA or zinc. Rat liver nuclear proteins (4 mg) were incubated with a ^{32}P-labeled 21 bp MRE-a* probe CTTTGCACCCGGACTG-GATC, in the presence of 100 μM EDTA (lanes 1 to 8) or 60 μM $ZnSO_4$ (lanes 9 to 16). Each series of reactions was performed in the presence of a range of concentrations of nonspecific competitor DNA as follows: series 1 to 8 and 9 to 16 each contained respectively 0, 0.03, 0.06, 0.12, 0.25, 0.5, 1, 2 and 4 μg of poly(dIdC). The major band at the bottom of the gel is the free probe; ZDC = zinc dependent complex.

Binding of ZAP to the MRE-a* probe was also tested at a range of zinc concentrations. As shown by the autoradiograph in Figure 3b and quantitated by densitometry in Figure 3c (upper line), the concentration dependence of ZDC formation on MRE-a* parallels that of MRE-a. Because the specific activity of the probes was similar and the free probe was in considerable excess over the complex (not shown), the relative intensity of the ZDC bands with the two probes indicates that the affinity of ZAP for MRE-a* is at least threefold higher than for MRE-a.

Sequence specificity of ZDC formation

In order to test comprehensively the target sequence requirements for specific binding of ZAP to DNA, mobility shift assays were performed using a series of variant MRE probes. Because of the higher affinity of ZAP for MRE-a* than for MRE-a, MRE-a* was used as the optimised binding sequence into which changes were introduced. MRE variants were produced containing every possible single nucleotide substitution throughout the 15 bp sequence of MRE-a*, and used as probes in gel retardation assays. This approach allows the zinc-inducibity of protein binding to the variant MREs to be assessed, and also detects the formation of novel protein:DNA complexes.

The relevant portions of the mobility shift gels are shown in Figure 5 (two different exposures of the gels are shown). Each probe was tested for binding in the absence and presence of zinc

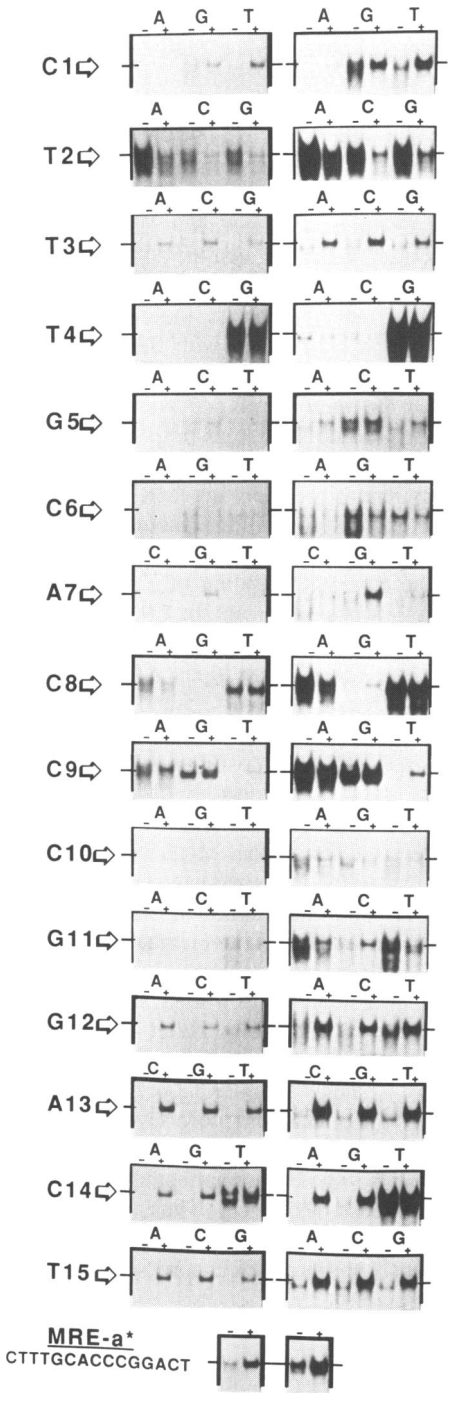

Figure 5. Testing MRE variants for zinc dependent protein binding. The MRE-a* 21 bp probe CCTTTGCACCCGGACTGGATC, and a series of variants containing single nucleotide substitutions at each of the underlined positions, were labelled with ^{32}P and used in mobility shift assays with nuclear proteins. Binding reactions contained either 500 μM 1,10-phenanthroline (an efficient chelator of zinc; tracks labelled −) or 100 μM $ZnSO_4$ (tracks labelled +). Each reaction contained 10,000 cpm (Cerenkov) of the appropriate probe. Low binding of the C1→A probe may be due to a hairpin bend at that end of this probe; the C1→T probe was 23 rather than 21 bp long, see Materials and Methods. Binding reactions and electrophoresis were performed at 0−4°C, to allow detection of weaker complexes. The zinc dependent complex obtained with the MRE-a* probe is shown at the bottom; the equivalent region of the gels using the variant probes are shown above. Each panel of 6 tracks shows the results obtained when the nucleotide indicated on the left was changed to each of the other three nucleotides. The series of panels on the right show a four-fold longer exposure of the autoradiograph than those on the left. The horizontal lines on either side of each panel indicate the relative mobility of the ZDC.

(indicated − and + above the tracks). The ZDC formed on the MRE-a* probe in this experiment is shown at the bottom of the figure. In order to enhance the detection of relatively weak complexes, the binding reactions were performed and the gels run at 0−4°C, leading to the detection of significant levels of ZDC (or a comigrating complex) even in the absence of zinc with MRE-a* and some of the variant sequences; however ZDC formation was still strongly induced by zinc. The remainder of the panels show the equivalent region of the gel using similar radioactive probes containing each of the variant MREs. The top row shows the results obtained with MRE variant probes in which C1 was changed to either A, G or T as indicated; the second row shows the effect of changes at T2, and so on.

Many of the MRE variants were able to form zinc-dependent complexes of identical mobility to the MRE-a* ZDC; variation in the intensity of these bands is indicative of variation in the affinity of ZAP for the target sequence. For example, most changes at positions G12 to T15 (downstream of the MRE core sequence) allow ZDC formation with an efficiency similar to MRE-a*, suggesting that these nucleotides are unimportant for ZAP binding. In contrast, most single nucleotide substitutions between T4 and G11 greatly reduce the intensity of the ZDC band, suggesting that ZAP binding is critically dependent on the sequence of this region. Note that the A7→G variant is identical to MRE-a, and as expected gives a zinc-induced band which is significantly weaker than that formed with MRE-a*. The variants with C or T at this position gave barely detectable ZDC bands, indicating that these changes cause a large reduction in the binding affinity. Changes at T4 and C6 resulted in undetectable levels of ZDC formation (the strong, zinc-independent band obtained with T4→G is discussed below; faint bands in the other tracks clearly migrate ahead of the ZDC).

Several of the probes formed retarded protein:DNA complexes which were unaffected or inhibited by zinc, some of which were more abundant than the MRE-a* ZDC. In some cases the mobility of such complexes was detectably different from that of the ZDC (e.g. C8→T, C9→G, C14→T; Figure 5 and unpublished results) suggesting that these complexes result from chance similarity of the MRE variant to the target sequences of other cellular DNA binding proteins; for example the C8→T substitution creates an almost perfect (7/8) match with the 'octamer' consensus sequence (*A*TTTGCAT, remaining discrepancy underlined), and so the constitutive band observed may be due to binding of the ubiquitous transcription factor OTF-1 (37).

Zinc-inducible complexes which apparently comigrate with the MRE-a* ZDC might nevertheless contain different protein species. In order to test directly whether the MRE variants bound the same protein as MRE-a* (ZAP), a competition experiment was performed in which unlabelled MRE variants were used to compete with radioactively labelled MRE-a* probe for ZAP binding. Oligonucleotides (38-mers) each containing two copies of the MREs were used as the competitors (see Materials and Methods); use of similar dimeric probes in mobility shift assays has shown that ZAP binds with the same affinity as to the monomeric probes, with no evidence for cooperativity due to the proximity of the two sites (unpublished results). The MRE competitors were added at approximately 250- or 1000-fold excess (50 or 250 ng) to the binding reactions containing zinc, prior to the addition of protein. The results are shown in Figure 6, in a format similar to that of Figure 5. Thus the ZDC formed on the MRE-a* probe without any oligonucleotide competitor is shown at the bottom of the figure, where it is also shown that

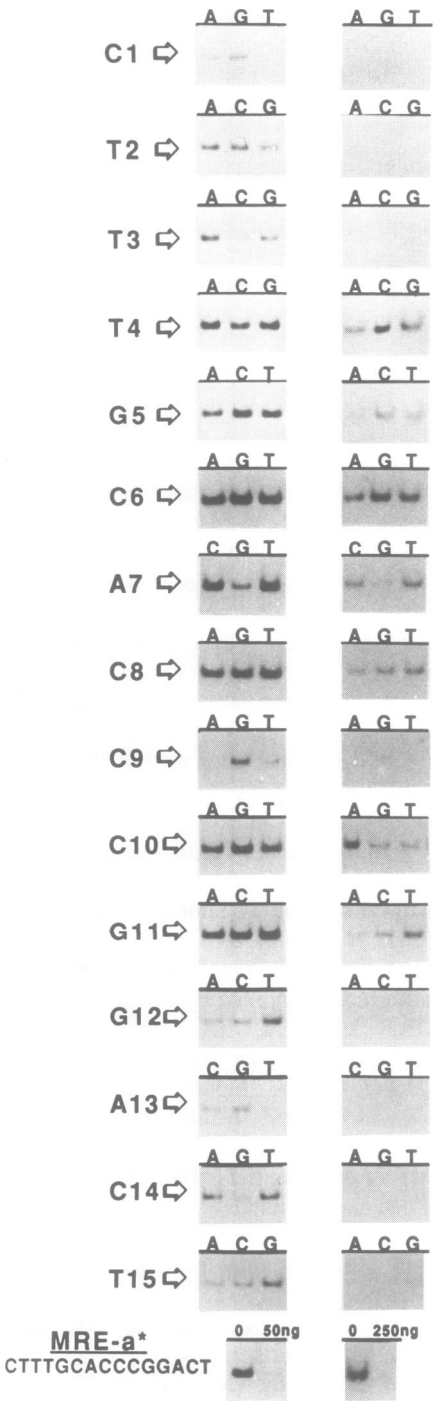

Figure 6. Competition of MRE variants with MRE-a*. Oligonucleotides (self-complementary 38-mers including 5'-GATC extensions, each containing two divergent copies of the MRE/MRE variant) were included in the binding reaction with 4 μg nuclear proteins, 1 μg poly(dIdC) and ^{32}P-labelled MRE-a* 21-mer probe (as used previously). All reactions contained 60 μM $ZnSO_4$. The ZDC formed on the MRE-a* probe without any MRE competitor is shown at the bottom (0) together with the effect of 50 and 250 ng of the unlabelled oligonucleotide competitor containing the homologous MRE-a* sequence, equivalent to approximately 250-fold and 1000-fold excess of unlabelled MRE. Each panel of three tracks above shows the effect when the nucleotide indicated on the left was changed to each of the other three nucleotides in the competing MRE; left hand series−50 ng; right hand series−250 ng oligonucleotide competitor.

250 ng of an oligonucleotide containing the identical MRE-a* sequence completely eliminates the ZDC band, whereas with 50 ng of the homologous competitor a faint band is just visible on the original autoradiograph.

It is apparent that most of the variants with changes in the MRE core region (T4 to G11) compete relatively poorly with MRE-a*, indicating that these nucleotide substitutions drastically reduce the affinity of ZAP for the sequence. Note particularly that T4→G is a poor competitor, indicating that the strong retarded complex observed with this variant in Figure 5 is due to binding of some protein other than ZAP. In contrast, the band obtained with the C9→A probe which comigrates with the ZDC (Figure 5) is apparently due to binding of ZAP, since this sequence competes at least as well as the homologous sequence against the MRE-a* probe (Figure 6). The nucleotide as position 9 is unspecified in the MRE consensus sequence, due to considerable variability at this position in naturally occurring MREs (18). One would therefore expect that all possibilities should be compatible with zinc regulated binding of the transcriptional activator, and this is seen to be the case with binding of ZAP. A ZDC band of moderate intensity is observed with the C9→G probe despite the presence of a strong zinc-independent band which migrates just slightly ahead; and C9→T also binds ZAP fairly well.

Considering the results of Figure 5 and Figure 6 together, it is apparent that any single nucleotide deviation from the MRE core sequence TGCRCNCG (positions 4 to 11) greatly reduces formation of the ZDC, although only changes at the T4 or C6 positions resulted in the complete absence of this zinc-inducible band in Figure 5. Outside the MRE core sequence, single nucleotide changes to the sequence had relatively minor effects. Although C1→A apparently inhibited ZDC formation, this is probably largely attributable to the fact that this particular probe was produced with a hairpin terminus (see Materials and Methods); similar gel retardation assays using dimeric MRE probes showed this variant to bind about as well as C1→G and C1→T (results not shown), in agreement with the competition assay (Figure 6). Changes at either T2 or T3 reduce the binding slightly, although the effect is no more severe than the transition A7→G which distinguishes MRE-a from MRE-a*. Formation of the ZDC was also relatively unaffected by changes 3' of the conserved MRE core sequence. Thus, the target sequence specificity of ZAP for binding to DNA is strikingly coincident with the previously defined MRE core sequence (18).

DISCUSSION

It is proposed that the MRE-binding protein detected in rat liver nuclear extracts (zinc-activated protein, or ZAP) is responsible for inducing transcription of metallothionein (MT) genes in that tissue in response to elevated concentrations of zinc. Gel retardation assays demonstrated a striking correspondence between the properties of ZAP and those of the MT gene regulator deduced from studies of both endogenous MT genes (17,36) and various MT/MRE promoter constructs transfected into tissue culture cells (15,16,21). Binding of ZAP to MRE probes is dependent upon the presence of zinc, and is maximally induced by 60−100 μM $ZnSO_4$, in good agreement with the concentration typically required to induce MT gene expression in cultured cells (36). In the presence of zinc, ZAP binds to MRE-a with much greater specificity than any other detected protein,

as judged by resistance to competition with poly(dIdC). The MRE consensus sequence allows either G (as in MRE-a) or A at position 7 (see Figure 1). Substitution of A at this position in the sequence of MRE-a (to give MRE-a*) resulted in approximately threefold greater binding of ZAP relative to MRE-a. Detailed analysis of the binding site preferences of the protein were tested using a series of single nucleotide variants of MRE-a*, and found to correspond extremely well with the previously defined MRE core sequence (18).

This detailed analysis of the sequence requirements for formation of the zinc-dependent complex (ZDC) provides a detailed picture of the relative importance of different basepairs within MRE-a* for binding of ZAP. While any single nucleotide deviation from the MRE core sequence TGCRCNCG greatly reduced ZAP binding, only changes at the T4 and C6 positions (underlined) completely prevented detectable ZAP binding (refer to Figure 1 for the numbering of basepairs within the MRE). This suggests that sequence-specific protein:DNA contacts involving these base pairs are required to anchor the protein tightly at one end of the core sequence, while allowing slightly greater flexibility over the remainder of the binding region. The relative importance of positions within the MRE alternates, suggesting that the protein has four principal regions of interaction with the MRE which are the major determinants of sequence specific binding at T4, C6, C8 and C10/G11 (the first two being more critical), while relatively minor interactions with the intervening base pairs 'fine tune' the affinity for variant MREs. At the less discriminatory sites G5, A7 and C9, there is again a gradation from end to end: G5 is strongly though not absolutely preferred over other nucleotides; A7 is about threefold better than A7→G while A7→C and A7→T bind extremely poorly. Requirements at C9 are quite lax, although ZAP binds with slight preference for A over C, with either being preferred to G or T at this position. The less stringent requirements at positions 7 and 9 are reflected in natural MREs, in which either purine is found at position 7 and all four nucleotides are represented with similar frequency at position 9, giving rise to the ambiguities of the consensus sequence. Outside of the MRE core, changes at positions C1 to T3 may cause minor reductions in binding efficiency; slight reductions in binding were also seen with some changes 3′ of the MRE core.

It is of interest to examine the extent to which determinants of specific binding to MRE-a* identified by this approach can predict the efficiency of MREs in metallothionein gene promoters *in vivo* (15–18). The data presented here have shown that MRE-a binding of ZAP is suboptimal due to the presence of G at position A7. The relatively poor efficiency of MRE-b of the mouse MT-I gene (Figure 1) may be attributed largely to the detrimental change G11→A. MRE-c is identical to MRE-a at all the important positions, and suboptimal changes at other positions may account for its marginally lower efficiency. MRE-d appears to be somewhat anomalous and will be discussed further below. MRE-e was ineffective at inducing transcription on its own (18), which may be attributed to the change G11→T. Finally, MRE-f contains the two highly detrimental changes C8→T and C10→G in addition to suboptimal nucleotides at other MRE core positions; it therefore seems improbable that ZAP could be responsible for the footprint observed *in vivo* (17) unless perhaps the binding is stabilised by protein:protein interactions. Direct assay of this sequence failed to detect independent regulatory activity (G.W. Stuart and P.F.S., unpublished results).

If binding to MRE-d followed the pattern described for MRE-a*, ZAP should bind less strongly to MRE-d than to MRE-a (A7→G), due to the detrimental change G11→C. However MRE-d is apparently the strongest MRE of the mouse MT-I promoter *in vivo* (15–18). One possible explanantion is that proteins other than ZAP are responsible for inducing transcription from MRE-d. It has been shown that Sp1 binds weakly to a site overlapping MRE-d both *in vitro* and *in vivo* (26,17), however it is doubtful whether this would play a role in inducible as opposed to constitutive expression. A more likely candidate is the factor MTF-1 identified in HeLa cell extracts, which binds to MRE-d in a zinc-dependent manner (26). Mapping of the MTF-1:MRE-d contacts by methylation interference showed that N7-methylation of guanines on the complementary strand at base pairs C13 and C15 of MRE-d interfered strongly with MTF-1 binding, whereas ZAP binding to MRE-a* does not appear to depend on specific protein:DNA interactions in this region. While this might support the existence of multiple regulatory factors, mobility shift assays using MRE-d either as a probe or in competition with an MRE-a* probe indicate that ZAP itself binds to MRE-d with an affinity intermediate between that for MRE-a and MRE-a* (unpublished results). This implies that the affinity of ZAP for MRE-d is not simply the product of the effects of single changes in MRE-a*; rather the changes must complement each other to allow an alternative pattern of specific contacts between ZAP and MRE-d. This interpretation allows that ZAP may be the rat equivalent of MTF-1.

The size of MTF-1 has not been reported; however a 108 kDa protein that binds MRE-d specifically has been detected in mouse fibroblast nuclear extracts by a protein blotting assay (25). Preliminary blots of the rat liver extracts using MRE-a* and selected variants as probes suggest a similar size for ZAP. A smaller protein of 74 kDa (MBF-1) has recently been purified from mouse fibroblasts (27) using affinity chromatography on a trout MRE, the core region of which matches the MRE-a* variant C9→A (which bound ZAP with high affinity; see Figures 5 and 6). MBF-1 was able to stimulate zinc-dependent *in vitro* transcription, although strangely footprinting of MBF-1 bound to the mouse MT-I promoter only detected binding to MRE-e (27), which was believed to have little or no regulatory activity (15–18). Further studies including purification and cloning of ZAP will be required to clarify its relationship with the other identified MRE-binding proteins and permit more direct studies of the protein:DNA interactions.

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REFERENCES

1. Posorke, L.H., Cohn, M., Yanagisawa, N. and Auld, D.S. (1979) *Bioch. Biophys. Acta* **576**, 128–133.
2. Hanas, J.S., Hazuda, D.J., Bogenhagen, D.F., Wu, F.Y.-H. and Wu, C.-W. (1983) *J. Biol. Chem.* **258**, 14120–14125.
3. Miller, J., McLachlan, A.D. and Klug, A. (1985) *EMBO J.* **4**, 1609–1614.

4. Klug, A. and Rhodes, D. (1987) *Trends Biochem.* **12**, 464–469.
5. Kadonaga, J.T., Carner, K.R., Masiarz, F.R. and Tjian, R. (1987) *Cell* **51**, 1079–1090.
6. Westin, G. and Schaffner, W. (1988) *Nucl. Acids Res.* **16**, 5771–5781.
7. Cousins, R.J. (1985) *Physiol. Rev.* **65**, 238–309.
8. Hamer, D.H. (1986) *Ann. Rev. Biochem.* **55**, 913–951.
9. Durnam, D.M. and Palmiter, R.D. (1981) *J. Biol. Chem.* **256**, 5712–5716.
10. Karin, M. and Herschman, H.R. (1981) *Eur. J. Biochem.* **113**, 267–272.
11. Oh, S.H., Deagen, J.T., Whanger, P.D. and Weswig, P.H. (1978) *Am. J. Physiol.* **234**, E282-E285.
12. Durnam, D.M., Hoffman, J.S., Quaife, C.J., Benditt, E.P., Chen, H.Y., Brinster, R.L. and Palmiter, R.D. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1053–1056.
13. Durnam, D.M. amd Palmiter, R.D. (1984) *Mol. Cell. Biol.* **4**, 484–491.
14. Karin, M., Haslinger, A., Holtgreve, H., Richards, R.I., Krauter, P., Westphal, H.M. and Beato, M. (1984) *Nature* **299**, 513–519.
15. Carter, A.D., Felber, B.K., Walling, M., Jubier, M.F., Schmidt, C.J. and ' Hamer, D.H. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7392–7396.
16. Stuart, G.W., Searle, P.F., Chen, H.Y., Brinster, R.L. and Palmiter, R.D. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7318–7322.
17. Mueller, P.R., Salser, S.J. and Wold, B. (1988) *Genes Dev.* **2**, 412–427.
18. Stuart, G.W., Searle, P.F. and Palmiter, R.D. (1985) *Nature* **317**, 828–831.
19. Seguin, C., Felber, B.K., Carter, A.D. and Hamer, D.H. (1984) *Nature* **312**, 781–785.
20. Karin, M., Haslinger, A., Heguy, A., Dietlin, T. and Cooke, T. (1987) *Mol. Cell. Biol.* **7**, 606–613.
21. Searle, P.F., Stuart, G.W. and Palmiter, R.D. (1985) *Mol. Cell. Biol.* **5**,1480–1489.
22. Anderson, R.D., Taplitz, S.J., Wong, S., Bristol, G., Larkin, B. and Herschman, H.R. (1987) *Mol. Cell. Biol.* **7**, 3574–3581.
23. Furst, P., Hu,. S., Hackett, R. and Hamer, D. (1988) *Cell* **55**, 705–717.
24. Seguin, C. and Hamer, D.H. (1987) *Science* **235**, 1383–1387.
25. Seguin, C. and Prevost, J. (1988) *Nucl. Acids Res.* **16**, 10547–10560.
26. Westin, G. and Schaffner, W. (1988) *EMBO J.* **7**, 3763–3770.
27. Imbert, J., Zafarullah, M., Culotta, V.C., Gedamu, L. and Hamer, D. (1989) *Mol. Cell. Biol.* **9**, 5315–5323.
28. Richards, R.I., Heguy, A. and Karin, M. (1984) *Cell* **37**, 263–272.
29. Schmidt, C.J. and Hamer, D.H. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3346–3350.
30. Gorski, K., Carniero, M. and Schibler, U. (1986) *Cell* **47**, 767–776.
31. Lichtsteiner, S., Wuarin, J. and Schibler, U. (1987) *Cell* **51**, 963–973.
32. Garner, M.M. and Revzin, A. (1981) *Nucl. Acids Res.* **9**, 3047–3060.
33. Fried, M. and Crothers, D.M. (1981) *Nucl. Acids Res.* **9**, 6505–6525.
34. Carthew, R.W., Chodosh, L.A. and Sharp, P.A. (1985) *Cell* **43**, 439–448.
35. Kadonaga, J.T., Jones, K.A. and Tjian, R. (1986) *Trends Biochem.* **11**, 20–23.
36. Yagle, M.K. and Palmiter, R.D. (1985) *Mol. Cell. Biol.* **5**, 291–294.
37. Fletcher, C., Heintz, N. and Roeder, R.G. (1987) *Cell* **51**, 773–781.