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Combinatorial Regulation by Promoter and Intron 1 Regions of the Metallothionein SpMTA Gene in the Sea Urchin Embryo

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The SpMTA metallothionein gene of the sea urchin *Strongylocentrotus purpuratus* is regulated developmentally, histospecifically, and by heavy-metal induction. The sequenced 5′ flank of the gene can be divided into proximal, middle, and distal regions, each containing a pair of metal response elements (MREs). Canonical 7-bp core sequences are present in all except the middle-region MREs c and d, which contain 1-bp mismatches. Metal-induced expression in transgenic blastulae was increased with each consecutive addition of the middle and distal regions to a chimeric reporter gene construct containing the proximal SpMTA promoter region. Reduced metal induction through point mutation of the distal MREs e and f indicated that the MREs themselves were largely responsible for the transcriptional increase. These activities were further enhanced by SpMTA intron 1, but not when a specific interior region of the intron had been deleted. The atypical MREs c and d did not support induction by themselves, i.e., when present alone with mutated proximal MREs a and b. However, in the presence of intron 1, they were able to substitute for the nullified MREs a and b in the promotion of metal-induced expression. This capability suggests, furthermore, that these atypical MREs, in addition to responding to an intron 1 region, participate cooperatively with the canonical proximal MREs.

The SpMTA gene of the sea urchin *Strongylocentrotus purpuratus* employs in its promoter a proximal pair of metal response elements (MREs), whose locations relative to each other and proximity to a TATA box are typical of metallothionein (MT) genes (20). Since MT genes generally have a multiplicity of MREs, which in the case of synthetic metal-responsive promoters can act synergistically (42), we have extended our analysis of the SpMTA gene here in order to identify any additional MREs, as well as other genic elements, that might regulate its metal-induced expression. Regulatory elements in addition to MREs have been localized only to the 5′-flanking regions of MT genes, even interdigitated with MREs. Although a pair of MREs can function as heavy-metal-dependent promoter elements in the presence of only a TATA box (43), basal level elements in MT promoters have been implicated as being required for constitutive expression and contributory to metal-dependent expression (24). While MREs can act as metal-inducible enhancers, even over a long distance (44), they are, in turn, modulated by other regulatory elements, e.g., through adjacent Sp1 sites or G boxes. In addition, the expression of some MT genes can be coordinated with other gene activities through common regulatory elements, such as those for glucocorticoid response (25).

The SpMTA gene is unique among MT genes in its intrinsic histospecific regulation (34). Hence, besides MREs, the SpMTA gene can be expected to contain regulatory elements that account for its expression being both developmentally scheduled (35, 50) and histospecifically regulated (34). Since its expression is restricted to the aboral ectoderm in sea urchin gastrulae and pluteus larvae, cultured in either the absence or presence of added heavy metal ions, metal induction does not override the histospecificity but appears, indeed, to be linked to it (34). Positive regulatory elements have been implicated in several genes expressed in the sea urchin embryo, and these together with negatively operative spatial-control elements have been identified in genes, such as those for cytoskeletal CyIIIa actin (48) and Spec 1 and Spec 2 troponins (16), which display the same aboral ectoderm specificity as SpMTA (34). Only one such element is indicated in the previously sequenced 0.6 kb of the 5′ flank of the SpMTA gene (20). However, we have identified a cluster of elements in the first intron of this gene with homology to the above-mentioned positive and spatial-control elements. Moreover, the absence of this intronic region from another MT gene, $SpMTB_1$, which is not aboral ectoderm specific (34), suggests that it should be studied as a potentially specific regulator of SpMTA gene expression.

To appreciate the full extent of promoter involvement in metal-induced expression, as well as the regulative participation of the first intron, we have obtained an additional 5′-flanking sequence and examined the activities in transgenic embryos of reporter gene constructs containing portions of 5′-flanking and intronic regions. Our observations indicate that the SpMTA gene is functionally bipartite, insofar as the 5′ flank promotes metal-regulated transcription, while the first intron amplifies this activity.

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MATERIALS AND METHODS

Sequencing and preparation of plasmids. The SpMTA MT λ206 clone from an *S. purpuratus* genomic DNA library (20) was the source of a previously restriction site-mapped subclone to be sequenced. A 1-kb region between *Acc*I and *Pst*I

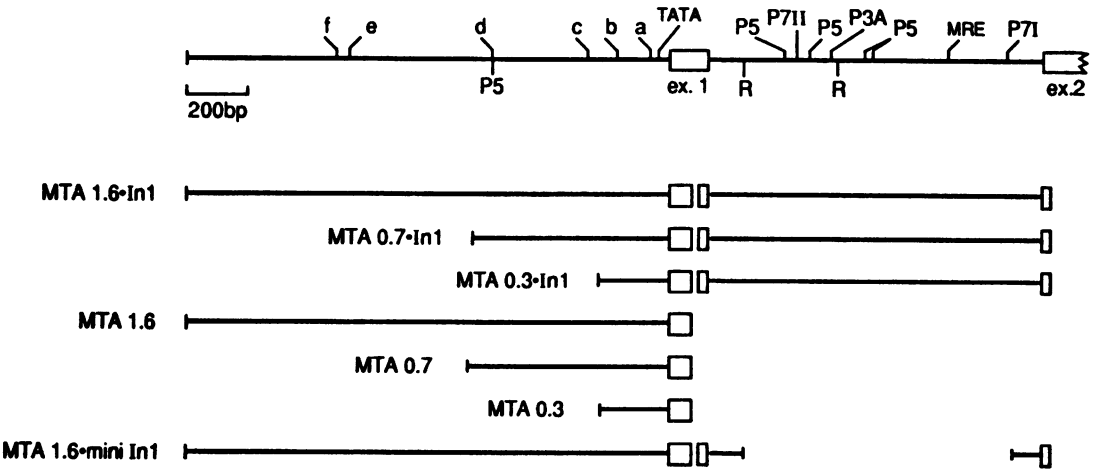

FIG. 1. Schematic representation of portions of the SpMTA gene used to generate chimeric constructs with reporter genes. The top line is a schematic of the portion of the SpMTA gene showing the 5'-flanking region with locations of MREs a through f and intron 1 between the first and second exons (ex.1 and ex.2). Also given are the locations of putative regulatory elements, defined by Thiebaud et al. (48). The SpMTA genic fragments in the chimeric constructs with reporter genes are diagrammed underneath with regions of the 5' flank, intron 1, and those portions of exons 1 and 2 which were included. Nomenclature refers to the extent of 5' flank and presence or absence of SpMTA intron 1. Several other constructs used in this study are not shown, including those with point mutations.

sites has been sequenced here by a chain termination method (51) through the use of priming oligonucleotides, which were specified by already-sequenced, downstream regions of the SpMTA gene. Plasmid DNA was prepared and purified through the use of the anion-exchange resin procedure of Qiagen (Duselldorf, Germany).

Chimeric constructs with the reporter CAT gene. The reporter chloramphenicol acetyltransferase (CAT) was derived from the herpes simplex virus thymidine kinase promoter construct pTK-CAT (obtained from R. J. Miksicek, German Cancer Research Center, Heidelberg, Germany) with a CAT splice-polyadenylation cassette (18) subcloned into the pUC8 plasmid. Regulatory sequences were inserted in place of the herpes simplex virus thymidine kinase promoter, which was removed as a *Hin*dIII-*Xho*I fragment, to yield the CAT-simian virus 40 (SV40)-pUC8 vector (CAT vector).

Into the *Hin*dIII and *Xho*I sites of the CAT vector were inserted a series of successively longer 5'-flanking regions of the SpMTA gene (Fig. 1), originating from the genomic clone λ206 (20). The 3' terminus common to these inserts was the *Xho*I site, 18 bp upstream from the SpMTA start codon. The retained portion of the first exon consisted of the 58 bp downstream from the transcription start point. The translation start codon of each construct was thus the first ATG of the CAT gene, in contrast to previous constructs, which expressed a partial MT-CAT fusion protein (20). MTA0.3 contained the 0.3-kb *Hha*I-*Xho*I fragment of λ206; MTA0.7 contained the 0.75-kb *Xho*I-*Acc*I fragment of λ206. These were derived, respectively, from constructs B and A of Harlow et al. (20). MTA1.6 contained the 1.6-kb *Xho*I-*Pst*I fragment of λ206, while MTA1.3 contained 1.25 kb of this fragment, lacking a 0.4-kb *Xba*I-*Pst*I portion.

Derivatives of these 5' promoter constructs, bearing the first intron of SpMTA, were prepared by inserting *Xho*I linker-bound copies of intron 1 into the *Xho*I site of either MTA0.3 or MTA0.7. The polymerase chain reaction-generated fragment of this 1.12-kb SpMTA intron, bracketed by 19 bp of exon 1 and 16 bp of exon 2, was generated with *Xho*I sites at both ends. In addition to intron 1, the resultant constructs contained 19 bp more of exon 1 than the constructs from which they were derived. These constructs with the intron in the normal orientation were termed MTA0.3-In1 and MTA0.7-In1, respectively. The intron 1-containing counterparts of MTA1.6 and MTA1.2, respectively designated MTA1.6-In1 and MTA1.3-In1, were obtained by inserting upstream *Hin*dIII-*Eco*RV fragments from either MTA1.6 or MTA1.3. A chimera with a mini-intron 1, MTA1.6-miniIn1, was constructed to resemble MTA1.6-In1, except for the deletion of an interior 0.9-kb region of intron 1. Two polymerase chain reaction-generated fragments, one from the −120 *Sac*I site to an appended *Sma*I linker site at 0.1 kb within intron 1 from the 5' splice junction and the other from a linker *Sma*I site, placed within intron 1 at 0.1 kb from the 3' splice junction, to the *Msc*I site in the CAT gene, were used to eliminate the interior 80% of intron 1.

SpMTA intron 1-containing constructs, having mutated MREs a and b, were prepared by utilizing construct a'b' (20), which contained the doubly mutated MREs a and b. A 0.74-kb *Hin*dIII-*Xho*I fragment of a'b' was used to replace a corresponding region in MTA0.7-In1 by inserting it in the 5.5-kb *Hin*dIII-*Xho*I fragment of MTA1.6-In1, to obtain a^-b^-MTA0.7-In1. Also, a^-ΔbMTA0.1-In1, a truncated version of a^-b^-MTA0.7-In1, was prepared by eliminating a 0.5-kb region, upstream from the *Sac*I site, situated between MREs a and b. The a^-ΔbMTA0.1-In1 construct retained only the mutated MRE a and lacked the other MREs.

Upstream core MREs e and f, at positions −1085 and −1125, respectively, were mutated separately to TGaAtTC and TGtAgAC (lowercase letters indicate replacements) by the method of Foss and McClain (13). These mutations were combined as a doubly mutated pair in MTA1.3 by use of the *Sph*I site between them to give e^-f^-MTA1.3. A 0.6-kb *Hin*dIII-*Eco*RV fragment from e^-f^-MTA1.3 was used to replace the corresponding region in MTA1.6-In1 to give e^-f^-MTA1.3-In1.

Chimeric constructs with the reporter firefly luciferase gene. Firefly luciferase constructs were based on pJD201 (10), into which the 1.2-kb *Eco*RI-bounded SV40 splice-polyadenylation cassette was added at the unique *Sma*I site (whereupon

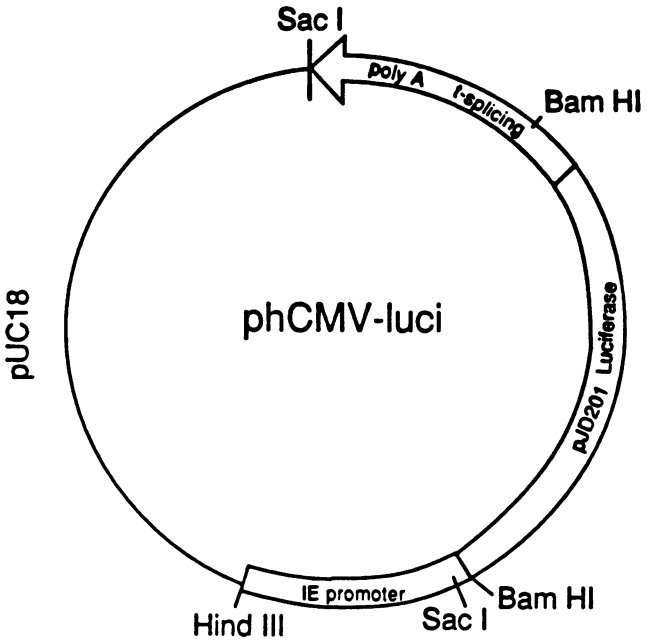

FIG. 2. The fusion gene hCMV-luciferase.

the *Eco*RI, *Sma*I, and adjacent *Kpn*I sites were destroyed) to give the firefly pUC18-luciferase-SV40 vector (luci-FF3). A region of the enhancer-promoter of the major immediate-early (IE) gene of the human cytomegalovirus (hCMV) as a construct with CAT (hCMV-CAT) was a gift from Jay Nelson (Research Institute of Scripps Clinic). This plasmid was used as a template for the polymerase chain reaction generation of a fragment which included positions −750 to +54 of the IE gene (6), as well as *Hin*dIII linkers at both ends, employing the primers 5'ATGCATAAGCTTGATC TATACATTGAATCAATA, in which IE gene positions −750 to −730 are underlined, and 5'TAGGTAAAGCTTCC TCTAGAGTCGGTGTCTTCT, in which IE gene positions +44 to +54 are underlined. This fragment was inserted into the *Hin*dIII site of luci-FF3 to produce the construct hCMV-luci (Fig. 2), which was used as a reference control construct.

An *Xho*I site was introduced into the *Bsm*I site at −90 of the luciferase cDNA (10) of luci-FF3 by interposing the half *Bsm*I-*Xho*I linkers 5'CATTCACTCGAGCAG and 5'GCTC GAGTGAATGCT. Into the 5.7-kb *Hin*dIII-*Xho*I fragment of this luci-FF3 derivative was inserted the 1.7-kb *Hin*dIII-*Xho*I fragment of the SpMTA promoter, obtained through partial digestion of MTA1.6, to produce the construct MTA1.6-luci. The MTA0.7-luci construct was made by deleting a 0.9-kb *Hin*dIII-*Acc*I fragment. The *Xho*I-site-bracketed intron 1 fragment (see above) was inserted in the correct orientation into the unique *Xho*I site of this plasmid to obtain MTA0.7-In1-luci.

Injection of chimeric genes into zygotes, culturing of embryos, induction with Cd(II) ions, and assays of reporter gene expression. For injection of *S. purpuratus* zygotes, the procedures of McMahon et al. (31) were modified as follows: eggs aligned on protamine sulfate-treated Falcon petri dish bottoms (60 by 15 mm) were dejellied by several passages through 180-μm-pore-size Nitex screening and settled twice. Eggs were fertilized following their positioning in rows. A borosilicate glass (inside diameter of 0.75 mm and outside diameter of 1.0 mm) with no filament (Sutter Instrument Co.) was used to pull microinjection pipettes by a Flaming-Brown micropipette puller (Sutter), and these were bevelled at an angle of 17° on a K. T. Brown type beveller (Sutter) prior to being siliconized in a dessicator under slight vacuum with hexamethyldisilazane. Plasmids, which had been linearized at unique restriction sites in the vector, together with an approximately twofold molar excess of *S. purpuratus* genomic DNA (sonication sheared to ~10 kb), were injected in a solution of 10% glycerol by use of an Eppendorf N_2-driven microinjector, a Zeiss IM inverted microscope with a KT temperature-controlled stage at 15°C, and a three-dimensionally controlled micromanipulator. After injection, the zygotes were cultured in synthetic seawater (SSW) (35) to the 24-h swimming blastula stage. In some cases they were cultured in SSW containing 50 μM ethylenediaminediacetic acid (EDDA). The blastulae were collected, washed in calcium-free SSW, and then quick-frozen and stored at −80°C. Embryos were lysed in 80 μl of a medium consisting of 25 mM Tris phosphate, 2 mM dithiothreitol, 2 mM EDTA, bovine serum albumin (1 mg/ml), and 10% glycerol. Lysis was performed by sonication for 3 s at an output setting of 1.5 (model W185D sonicator; Heat Systems, Plainville, N.Y.), generating 40 to 50 Watts. CAT activity was assayed in 50 μl (18) as described previously (20). Luciferase activity in 10 or 20 μl of the lysate was assayed (10) by addition to 380 μl of a solution containing 0.1 mM ATP, 10 mM $MgCl_2$, and 25 mM Tris phosphate, pH 7.8. Into this solution was injected 100 μl of solution containing 10 μM luciferin, 10 mM $MgCl_2$, and 25 mM Tris phosphate, pH7.8, and the luminescence was measured with a Monolight 2101 luminometer (Analytical Luminescence Laboratories, San Diego, Calif.). To obtain uniformity, eggs from only one female were used for each experimental set; about 200 eggs were injected per experimental point in order to ensure a detectable level of reporter gene expression for the SpMTA promoters.

Quantification of chimeric CAT reporter gene expression based on the simultaneous expression of a reference chimeric construct containing the hCMV enhancer promoter and the luciferase gene. We have quantified the activities of transgenes by coinjecting a reference transgene bearing a different reporter. Sonication, described above, allowed recovery of both reporter gene products, CAT and luciferase, in high and invariant yield. In contrast, 50% of luciferase was lost through freeze-thawing (31); 100% of luciferase was lost by 0.1% sodium dodecyl sulfate treatment (38); and 96% of CAT was lost when 1% Triton X-100 was included in the lysis medium. That SpMTA-CAT and hCMV-luciferase gene constructs were expressed independently, i.e., without an effect of one promoter upon the other, was shown by the proportionality between their expression ratio and input ratio when either was varied within limits and, furthermore, by a constant level of hCMV-luciferase expression in the face of a severalfold change in the activity of the SpMTA-CAT construct through metal induction. When a constant amount of hCMV-luciferase was coinjected with variable amounts of CAT gene construct, the CAT reporter expression relative to reference luciferase was proportional to the dosages over a specific input range (see below, Fig. 6).

Nucleotide sequence accession number. The nucleotide sequence is an update of GenBank/EMBL accession number M30606.

GATCCTAAGTCGACTGAGGTTCACGGATCAGGTTGATAAACTTGCAGCTCAGCCATGGAGGTATGCATCAATCCTTGTATGATAAAAAATGTACTCGATG -1521

ACCCCGTGAAGAACGAAGGCGGCGGGCAAAAATATACAGGCATTTTAAGAAGTCCAGAGCTACATGAACATCTTGGTTATAAATATATCCTGCTGTGGAC -1421

TGCGAATTGAATGATGTTTATAATCCTGTTTTTACCTTTGGAAATTGCAGTAATAATAGAATGTAATACCTCCAACATTTGGTAAAGAGTTTGGAGTTGG -1321

TATTTCGTATGATTATTCAGCGAATTTTAGAAAACAAGAGCAGTTCAAAAATATTTATCCATCTAGAAGCCACACAAGACCGGTCACATGATTCAACCCA -1221

GCCAAACTCAGAAAAGCTGCCCGCACATAAGTGTACTGTACGTGTCACGTATTTAAATAATCTTGAATATTCAAACTATTGTTATATTATTCGTGTGCAA -1121
MRE_f

TAATGAGGACGAGCATGCAAGCGCTACTCGAGTGCAAACCCAACCACCTCTAGTCTCTACTTAAGGTGTTTAAATCTTTATCTCAAAGTAGACAGAAAAG -1021
MRE_e

ACGTCATAATCATATTTGTATCACCTCCCCCCTCTCCCCTTGGGTAGGGAAGGCGAAATTTGAGAAAACAATAGAAATTGAACATTAATAATGTATTAAT -921

AATGTTTATGCTCCATTTCATGCATTTCGTGTGAAACAACGAGTTAAAAATATGCATGCAGTAAATCCTTTCGTCTCCACTCTGGGTCAATCGTCAGGGG -821

CAGATCCAGGATTCATTTGAAATGAAATCGAGGAGGGAGGGGGGGGGGGGAGATTTCCACTGATGCCTGGTGTGTAGCACTATGGCAATTTGACTGGGGG -721

TAAGTGTCTCTAATACGCCCTATAGGCAAATATTGACAGGTCTACGTAATT -670

FIG. 3. Sequence of the 5′-flanking region of the SpMTA MT gene from −0.6 to −1.6 kb. The MREs e and f are in boldface type and labelled. Poly-Y and poly-R tracts are underlined.

RESULTS

Nucleotide sequence of an upstream 5′-flanking region of the SpMTA gene. To determine whether additional regulatory elements might be detected beyond the previously sequenced 0.6 kb of proximal promoter region (20), we have sequenced an additional 1-kb upstream region (Fig. 3). This upstream sequence contains a pair of MREs, e and f, 30 bp apart at −1085 and −1122, as well as stretches of 24 purines and 18 pyrimidines at −768 and −980, respectively. Therefore, the SpMTA promoter (Fig. 1) contains six MREs; two MRE pairs, the proximal a and b and the distal e and f, have the TGCRCNC core sequence, which has been demonstrated to be optimally functional in mammalian MTs (9, 43), while both intermediately located MREs, c and d, have a single mismatch. The upstream region contains none of the putative regulatory elements defined by Thiebaud et al. (48), i.e., the sites for positive amplification factors P5 and P7I and the negative spatial factors P3A and P7II. We have taken as consensus sequences for these sites those suggested by Thiebaud et al. (48) for P3A (YNYGCGCW), P7II (STWAC CCTYW) and P7I (TTGTNSKYAY) and have extended the core of their P5 consensus to TTCATKSTNKYNTNW on the basis of an enlarged sample of 16 sites, which includes similar sites in the SpMTA (20) and SpMTB$_1$ (34) genes. Whereas we can identify only one of the putative amplitude and spatial regulatory sites in the 5′ region of the SpMTA gene, a P5 site at −595 (identity of 14 of 15 positions), a cluster of several such regulatory sites is present in the first intron of SpMTA: P5 at +385 (14 of 15 positions), +466 (14 of 15 positions), and +655 (14 of 15 positions); P3A at +526 (9 of 9 positions); p7II at +416 (8 of 9 positions); and p7I at + 1100 (9 of 10 positions) (Fig. 1). Also present in intron 1 is a single consensus MRE motif at +915.

Metal-induced expression is increased by successive additions of 5′-flanking regions to the SpMTA promoter and further enhanced by intron 1. Our results (20) indicated that the proximal 0.3-kb flank of the SpMTA gene could be considered a core metal-inducible promoter, consisting of a TATA box and the proximal pair of MREs a and b. We showed, furthermore, that the adjacent 0.4-kb upstream region increased this core activity. In Fig. 4, we have extended this series of 5′-flanking additions to a CAT reporter gene by adding to this 0.4-kb middle region an adjacent 0.9-kb distal region and by testing their metal-induced expression in transgenic blastulae. The employment of dosages of 250 and 2,000 molecules injected per zygote gave essentially similar relative activities. Relative to the unit activity of the 0.3-kb core promoter, we observed a twofold increase with the 0.7-kb 5′-flanking region and a further twofold increase to four times the core promoter activity with the 1.6-kb 5′-flanking region. On the basis of the effects on metal-induced expression of multiple MREs in a synthetic promoter (42), it is a reasonable hypothesis that these stepwise increases are attributable to the pairs of MREs in the middle and distal regions of the 5′ flank.

The striking difference that we have described between the first introns of the SpMTA and SpMTB$_1$ genes, particularly the insertion of potential regulatory DNA elements in intron 1 of SpMTA (20, 34), led us to examine whether this intron might influence gene activity. We found that the SpMTA intron 1, positioned between the promoter and the CAT gene, amplified the activity of the core promoter twofold but that the activities of both the 0.7-kb and 1.6-kb promoters were amplified fourfold. Consequently, the intron enhances their activities to levels, respectively, 8 and 16 times that of the core promoter. Two components appear to constitute this quantitative regulation. (i) Twofold increases occur with the addition of each region in the absence of intron 1, and a twofold increase also occurs with the addition of the distal region in the presence of intron 1. It could be surmised that these increases are attributable to the MRE pair in each region. (ii) While intron 1 enhances the 0.3-kb core promoter activity twofold, a more substantial enhancement, fourfold, is applicable when the middle region is present, in either the 0.7- or 1.6-kb promoter. It could therefore be hypothesized that the middle region is the target of intron 1 influence.

To test whether the effect of intron 1 was reporter specific, we reversed the SpMTA and hCMV reference genes. When 500 molecules of either MTA0.7-luciferase or MTA0.7-In1-luciferase were injected together with 2,000 molecules of reference hCMV-CAT, the MTA0.7 promoter was expressed at a level of 61 fg of luciferase per μU of CAT, compared to 175 for MTA0.7-In1. This threefold increase in

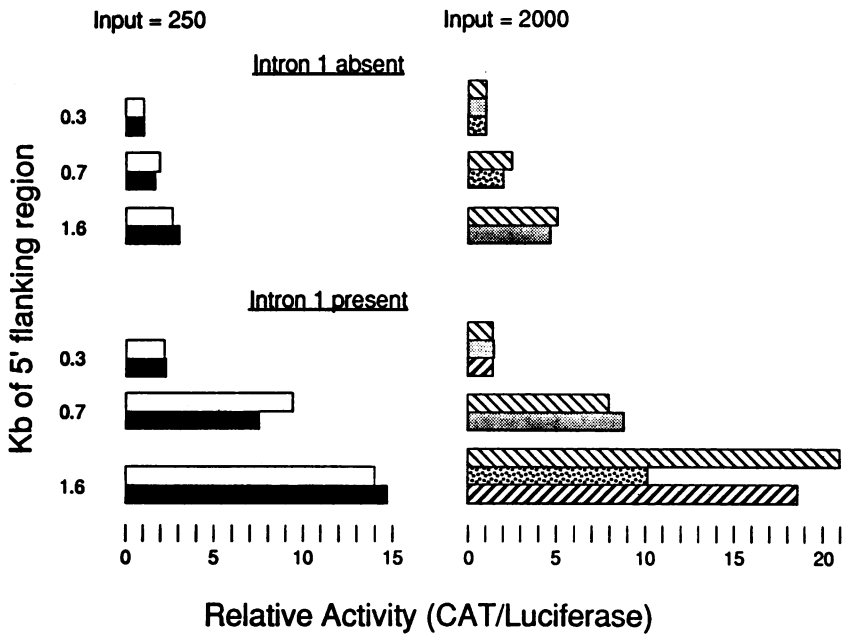

FIG. 4. Effects of varied portions of the 5'-flanking region and intron 1 on metal-induced expression of the SpMTA gene. Zygotes were injected with 1 pl containing 1,000 molecules of hCMV-luciferase and either 250 or 2,000 molecules (as indicated) of CAT-reporter gene constructs with the indicated lengths of SpMTA 5'-flanking region in the absence (constructs MTA0.3, MTA0.7, and MTA1.6) or presence (constructs MTA0.3-In1, MTA0.7-In1, and MTA1.6-In1) of SpMTA intron 1. Embryos were cultured in SSW to the 24-h blastula stage and then induced for 5 h with 200 μM cadmium acetate. Activities relative to that of the simplest construct, MTA0.3, were calculated from values of CAT activity per luciferase activity. Each bar pattern represents an individual batch of eggs.

activity was in agreement with that obtained for the CAT reporter and indicates that the effect of intron 1 is not a function of the reporter gene.

Effect of a deletion in SpMTA intron 1 on amplified expression. Stimulated expression has been reported by heterologous introns positioned 5' to the CAT gene, even when the 3' small SV40 intron was already present (3, 7, 11). To test whether the SpMTA intron 1 might be operating nonspecifically in its stimulation of the SpMTA promoters, we utilized MTA1.6-miniIn1, which is a derivative of MTA1.6-In1 but lacks the interior 0.9 kb of intron 1, retaining approximately 0.1 kb at each intron terminus. Since this construct expressed its reporter gene (Fig. 5), it had to have been transcribed and its mini-intron had to have been spliced out. The capability of being spliced would also have been predicted from its retention of a consensus lariat target site (45) and a polypyrimidine track (47). Furthermore, a construct with the intron in the opposite orientation did not express a CAT reporter gene (not shown). The deletion of the internal region of intron 1 reduced expression to the level of the construct which lacked intron 1 entirely, i.e., to that of MTA1.6 (Fig. 5). Therefore, we cannot attribute the amplification of the SpMTA gene simply to the presence of a nonspecific intron placed 5' to the reporter gene but must also attribute it to the content of that intron.

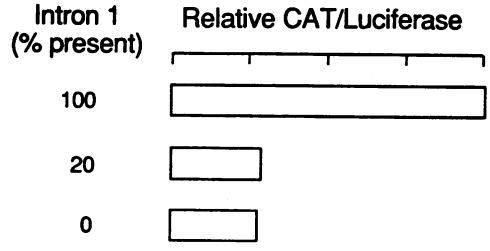

FIG. 5. Effect of deleting the interior of SpMTA intron 1. Zygotes were injected with 1 pl containing 1,000 molecules of hCMV-luciferase and 500 molecules of CAT construct, containing 100% of intron 1 (MTA1.6-In1) or 20% of intron 1 (MTA1.6-miniIn1) or completely lacking intron 1 (MTA1.6). Embryos were cultured and induced with Cd(II), and reporter genes were assayed as for Fig. 4. Values are averages from two batches of eggs.

Metal-induced and noninduced expression as a function of dosage of injected SpMTA genes. The CAT construct with the most complete representation of the SpMTA gene used here, MTA1.6-In1, was injected at a nominal volume of 1 pl to administer dosages of 50 to 4,000 molecules per zygote together with a constant dose of 1,000 molecules of hCMV-luciferase. Embryos were cultured for 24 h in SSW and then fully induced in excess Cd(II) or alternatively cultured in chelator (50 μM EDDA) (Fig. 6). Expression in the metal-induced blastula was proportional to gene input at values less than the sharp maximum displayed at 450 MTA1.6-In1 molecules per zygote. In this low input range, the apparently constitutive expression observed in chelator-cultured blastulae was negligible, at <1% of the metal-induced expression. However, with increased dosage this noninduced expression approached 20% of the induced expression and displayed a broad maximum around an input of ~2,000 molecules. An input dose-response series for CyIIIa actin-CAT, similarly utilizing the hCMV-luciferase reference activity (Fig. 6), showed expression proportional to construct input up to about 1,000 molecules per zygote and a gradual increase to a broad plateau just beyond 2,000 molecules per zygote, consistent with curves previously described by the method of normalization to reporter gene titer (14, 29), and similar to noninduced SpMTA expression.

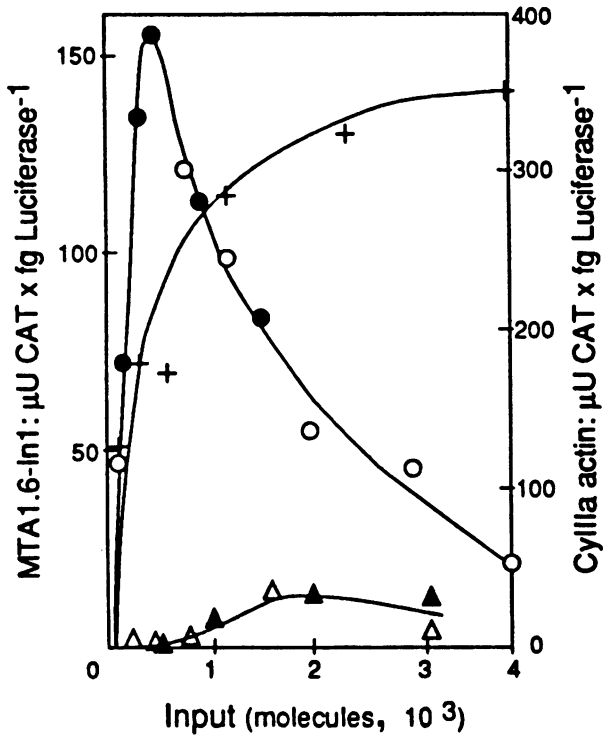

FIG. 6. Reporter gene activities as a function of dosage of either SpMTA or CyIIIa constructs. Zygotes were injected with 1 pl containing the indicated input number of molecules of CAT constructs, either MTA1.6-In1 or CyIIIa actin, together with 1,000 molecules of hCMV-luciferase. Those injected with MTA1.6-In1 were either cultured in SSW for 24 h and then incubated in 400 μM cadmium acetate for 5 h (○ and ●) or cultured for 29 h in 50 μM EDDA (△ and ▲), where open and closed symbols represent different experiments normalized to common points. Embryos injected with CyIIIa actin-CAT (+) were cultured for 29 h, and one of two experiments is represented. Embryo extracts were assayed simultaneously for CAT and luciferase activities.

A considerably smaller number of SpMTA transgenes are required for maximal metal-induced expression than for constitutive expression. This result suggests that either (i) the titer of factors involved specifically in metal induction is appreciably lower than that of factors supporting constitutive expression or (ii) the transgenes are utilized at a reduced efficiency because of the low, unaugmented intracellular metal ion concentration in the presence of chelator, thereby requiring a higher input for maximal expression. The former explanation presumes that the basal or constitutive expression is not induced by metal ions. The latter allows the possibility that even basal expression of the SpMTA gene is fully dependent upon metal induction (see Discussion).

Metal-induced expression by SpMTA promoters containing mutated MREs. The low constitutive activities over a wide input range for the 1.6-kb SpMTA promoter in the presence of intron 1 (Fig. 6), as well as the substantial metal induction compared with basal levels for the 0.7-kb SpMTA promoter region in both the presence and absence of intron 1 (Table 1), indicate that the intron 1-elevated activity observed in Fig. 4 is attributable predominantly to metal induction rather than to an enhancement of basal activity. In order to explore the parameters of this inductive activity further, the core sequences of the proximal MREs a and b at −46 and −146 were mutated to TGCcCAa and TGatCAC, respectively.

TABLE 1. Mutated MREs in the presence and absence of SpMTA intron 1[a]

5′ flank (kb)	Intron 1	MRE(s)	Cd	μU of CAT/ embryo	fg of luciferase/ embryo	μU of CAT/fg of luciferase	Ratio (+Cd/−Cd)
0.7	+	abcd	−	0.086	1.10	0.08	9.3
			+	0.837	1.16	0.73	
0.7	−	abcd	−	0.020	0.63	0.03	10.0
			+	0.270	0.79	0.34	
0.7	−	a^-b^-cd	−	0.013	0.32	0.04	0.5
			+	0.018	0.87	0.02	
0.7	+	a^-b^-cd	−	0.133	1.41	0.09	4.6
			+	0.445	1.04	0.43	
0.1	+	a^-	−	0.380	1.40	0.27	0.8
			+	0.338	1.61	0.21	

[a] Zygotes were injected with 1,000 molecules of hCMV-luciferase and 500 molecules of CAT constructs having the indicated lengths of 5′ flank with all wild-type MREs (MTA0.7 or MTA0.7-In1), wild-type MREs c and d and point-mutated MREs a^- and b^- (a^-b^-MTA0.7 or a^-b^-MTA0.7-In1), or only a^- (a^-MTA0.1-In1) and containing (+) or lacking (−) SpMTA intron 1. Embryos were cultured in SSW with 50 μM EDDA to the 24-h blastula stage and then either continued in this medium to measure basal expression or induced for 5 h in 200 μM cadmium acetate in SSW to measure induced expression. Values are averages from duplicate determinations.

When these mutations were present in the 0.7-kb promoter lacking intron 1, metal induction was eliminated (Table 1), in agreement with our previous results (20). The 0.5 ratio of induced/basal expression indicated that expression was actually reduced by the metal ions. In addition to MREs a and b, two other elements, MREs c and d, which depart by a single mismatch from the highly conserved MRE 7-bp core, are within the 0.7-kb flanking region at positions −253 and −600. These additional MREs apparently do not rescue metal-induced expression. The addition of intron 1 to the 0.7-kb promoter bearing mutated MREs a and b permitted metal-induced expression and thus potentiated the functionality of the otherwise defective MREs c and d. A construct entirely lacking in promoter wild-type 5′ MREs and retaining only a 0.1-kb 5′-flanking region with the mutated MRE a, the TATA box, and intron 1 also did not display metal-induced expression; indeed, it was inhibited by Cd(II) treatment. This lack of metal induction, moreover, suggests that the single MRE in the intron is not operative in metal induction by itself.

The core sequences of the far upstream MREs e and f at −1085 and −1122 were mutated to TGaAtTC and TGtAgAC, respectively, and their contribution to metal induction was analyzed in constructs containing 1.3 kb of promoter together with intron 1 (Table 2). An average ratio of 2.7 was obtained for the activities of the construct containing intact wild-type MREs a, b, c, d, e, and f compared with those of the construct retaining only wild-type MREs a, b, c, and d. A similar ratio was obtained in Fig. 4 when the activities of the 1.6- and 0.7-kb promoters containing these respective sets of wild-type MREs were compared. Therefore, the upstream MREs e and f contribute to metal-induced SpMTA expression and may account for most of the stepwise increase in activity resulting from the addition of the distal 5′-flanking region which contains them.

DISCUSSION

Successively increased metal inducibility attributed to a series of MREs, both canonical and atypical. The proximal

TABLE 2. Mutation of upstream MREs[a]

Expt	MREs	μU of CAT/ embryo	fg of luciferase/ embryo	μU of CAT/fg of luciferase
1	abcdef	3.02	2.21	1.37
	abcde$^-$f$^-$	1.95	3.52	0.55
2	abcdef	0.332	0.355	0.94
	abcde$^-$f$^-$	0.046	0.147	0.31

[a] Zygotes were injected with 1,000 molecules of hCMV-luciferase and 500 molecules of the CAT construct with SpMTA intron 1 and the promoter regions containing the indicated wild-type MREs or point-mutated MREs e$^-$ and f$^-$ in their place in constructs MTA1.3-In1 and e$^-$f$^-$MTA1.3-In1, respectively. Blastulae were developed in SSW and induced for 5 h in 200 μM Cd(II). Expression was measured as in Table 1.

0.3-kb flank of the SpMTA gene can be considered a core metal-inducible promoter, consisting of a TATA box and the proximal pair of MREs, a and b. An upstream 0.4-kb extension of this core promoter into a region containing the pair of atypical MREs, c and d, doubles the metal-induced promoter activity; a further upstream 0.9-kb extension into a region containing the pair of perfect MREs, e and f, further doubles the promoter activity. That these successive increases might be attributed largely to the involvement of the MRE pair in each of the additional upstream segments could be reasonably posited on the basis of a previously noted enhanced metal inducibility of a mouse MT promoter by successively added pairs of synthetic MREs (42). However, a corollary dependence of fully metal-induced expression on the integrity of all MREs within a promoter has not been reported. In the case of the SpMTA gene, mutation of the MREs e and f prevented the stepwise increase in metal induction that would have been attained by the addition of an upstream region containing them. It is thereby suggested that these MREs are the DNA elements responsible for this increase. Although the atypical MREs c and d in the midregion of the promoter could not act alone and thus rescue the activity of a promoter with mutated MREs a and b, it is nevertheless likely that they are responsible for the increased induction produced by the addition of the middle promoter region to the proximal region containing wild-type MREs a and b. The inference here that they could act synergistically with viable MREs a and b to increase inducibility could be extrapolated from their apparent cooperative activity with intron 1 (discussed below). While the predominantly metal-inductive function of the 5′ flank of the SpMTA gene would thus be attributed largely to a functioning series of MRE pairs, it cannot be excluded that other regulatory motifs, such as the P5 element, overlapping MRE d in the middle region of the promoter, play a role in the stepwise increase in activity.

The cryptic involvement of MREs c and d was revealed through the inclusion of intron 1. Although incapable of functioning by themselves in a construct lacking intron 1, these MREs were rendered functional through the inclusion of intron 1. This hidden potential is reminiscent of the relationship among the operator sites of the *lac* operon, in which the pseudo-operators O2 and O3 cannot support repression by themselves but can do so by interacting with operator O1 through the tetrameric *lac* repressor (39). While a multiplicity of MREs with optimally functional 7-bp cores (9, 43) is the rule for MT promoters in general (19, 49), a large number of MT genes have some MREs with single-base mismatches; indeed, MRE f of the mouse MTI gene has two mismatches and yet appears to be a protein-binding site (32). It might be posited that such atypical MREs, incapable of functioning independently, may nevertheless have their factor-binding capacities enhanced by combinatorial interactions with other regulatory sites. The parameters of tolerance that govern the participation of the components of a gene appear to be broader than the properties of each isolated component would predict. This admonition may apply either to the pseudo-operators of the *lac* operon or to atypical MREs with respect to interactions involving their canonical counterparts.

Requirement for metal induction. We showed previously (51) that transcription of the endogenous SpMTA gene and the concentration of SpMTA mRNA were reduced to undetectable levels in embryos cultured in EDDA-treated sea water medium (SSW). We noted also that a detectable amount of SpMTA mRNA could be made to accumulate by adjusting the chelater-treated SSW to a net concentration of 1 μM Zn(II) (51), a level of copper and zinc ions that previous measurements (35) indicated could be present in untreated SSW. It could thus not be excluded that most of the constitutive SpMTA gene activity by embryos in untreated SSW was attributable to induction by heavy-metal ions in the seawater medium rather than to an intrinsic basal expression. The micromolar concentrations of EDDA employed to chelate heavy metal ions would have a negligible effect on the millimolar levels of Mg(II) and Ca(II) ions in the SSW (35). It is also important to note that development of embryos in SSW treated with up to 100 μM EDDA is completely normal (33, 36, 51), and therefore the exogenous chelator could not deprive embryos of essential, intracellular zinc or copper ions. The role of noninduced expression of the SpMTA gene is further diminished by our present observation that transgenic expression in chelator is vanishingly low at an SpMTA construct dosage that could otherwise elicit maximal metal-induced activity. Indeed, it cannot be excluded that in chelator-treated SSW, the approximately fivefold increase in construct dosage required to attain appreciable expression facilitates utilization of endogenous metal inducers rather than optimization of factors engaged exclusively in constitutive, noninduced expression. While a complete dependence of SpMTA expression on metal induction would seem to contrast with mammalian genes, which have basal regulatory elements as well as hormone response elements (19, 49), an absolute requirement for inductive heavy-metal ions, of possibly low concentration, has not been excluded for any promoter containing operative MREs.

Characteristics of intron-directed enhancement. Diverse modes of action have been proposed for regulatory elements in introns. These include intronic enhancers in genes for immunoglobulin (2, 17, 26), δ-crystallin (21), collagen (4, 5, 28, 41, 46), and keratin (40); intronic sites for transcription arrest in genes for c-Ha-*ras* (30), adenosine deaminase (8), and c-*myc* (1); and intronic sites responding to physiological signals, such as the serum response element in the β-actin gene (15, 37), the spatial and temporal control elements in the engrailed gene (27), and histospecific control elements in several genes, operating through the enhancement of promoter activity (17, 21, 41) or through alternative splicing (12, 22, 23, 50). However, effects of introns in plasmid constructs are not all readily explained in terms of either RNA processing or promoter enhancement, nor are they necessarily free of posttranscriptional peculiarities of reporter gene expression. Several genes are more efficiently transcribed than their intronless cDNA counterparts in transgenic mice but not in transfected tissue culture cells; however, this effect could not readily be attributed to RNA processing or en-

hancers (7). Also, the positioning of introns could be influential, with 5′ introns being considerably more stimulatory than 3′ introns on chimeric CAT and β-galactosidase (11). Moreover, when included with the human thrombospondin promoter, the first intron of this gene amplifies CAT, but not growth hormone, reporter gene expression (3). Indeed, introns in the 3′-untranslated region can inhibit chimeric CAT and β-galactosidase gene expression (11).

The intron 1-lacking constructs of the SpMTA promoter double in activity with each addition of an upstream region containing a pair of MREs, generating an approximate activity series of 1:2:4. The inclusion of intron 1 superposes another doubling with each of these additions, generating an approximate activity series of 1:4:8. It would seem, then, that an increase in the effectiveness of bringing into play an additional pair of MREs is the principle quantitative consequence of intron 1 inclusion. Since the addition of the distal region containing MREs e and f produces a doubling in both the absence (2:4) and presence (4:8) of intron 1, we can deduce that the target for the intron effect resides in the middle promoter region. This 0.4-kb region, enlisting the intron, quadruples rather than doubles the level of expression. Our findings do not indicate the nature of such a hypothetical target site in this region. By demonstrating that deletion of the 0.9-kb interior region of intron 1 reduces the expression level to that of a construct lacking intron 1, we have established that the enhancement cannot be attributed simply to the presence of a generic 5′ intron and its attendant nonspecific effects on reporter gene expression (3, 7, 11). Within this deleted region of intron 1 reside several sequence motifs strongly homologous to sites (48) which have been implicated in the regulation of members of the histospecific gene set to which SpMTA belongs and are thereby potential regulatory sites for this gene. In conclusion, our results suggest that the SpMTA gene is composed of two functionally distinct operating components: (i) a promoter whose heavy-metal ion-dependent activity is sustained through the cooperative interaction of a series of MRE pairs and (ii) an intron with regulatory elements that enhance this expression.

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