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Structure of an Ectodermally Expressed Sea Urchin Metallothionein Gene and Characterization of Its Metal-Responsive Region

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The metallothionein-A gene in the metallothionein gene family of the sea urchin *Strongylocentrotus purpuratus* (SpMTA gene) was sequenced and found to contain three coding exons plus a 3′ entirely noncoding exon. Putative α and β MT domains were encoded, by its exons 2 and 3, respectively, in reverse of the order in vertebrate metallothionein genes. The SpMTA promoter was characterized through the expression of recombinant constructs containing various portions of the proximal 678-base-pair (bp) 5′-flanking region of the SpMTA gene. Zygotes injected with constructs were cultured to the blastula stage in the presence of a heavy-metal chelator and then incubated in the presence or absence of cadmium. The longest constructs were expressed only when heavy-metal ion was present. Two putative metal-responsive elements (MREs a and b) within 240 bp of the transcription start site resembled mammalian MREs in their critical 8-bp cores (TGCRCNCS) and in their locations relative to each other and to the TATA box. Elimination of activity by site-specific mutations in MREs a and b, separately or in both, identified them as metal regulatory elements. Thus, MRE recognition in this invertebrate resembles that in vertebrates. Upstream sites with single-mismatched MREs neither acted as MREs nor amplified the activity of MREs a and b. The SpMTA, Spec1, and CyIIIa actin genes, which have the same ectodermal specificity, have common DNA elements at relatively similar locations in their promoter regions; however, these elements are insufficient in themselves to promote gene expression.

Metallothioneins (MTs) are small cysteine-rich proteins that bind to heavy metals. The induction of MT genes by heavy metals has been studied in a variety of systems as a model of gene regulation (22, 45). *cis* regulatory metal-responsive elements (MREs) have been identified and implicated in the induction of MT gene transcription through gene transfer experiments; of the 12 to 15 nucleotides deemed to constitute MRE regions (53, 59), only the eight-nucleotide core, TGCRCNCS (where R is A or G and S is C or G), needs to be conserved (12, 50). Nevertheless, the functionality of MREs with divergent core sequences is suggested, particularly in the case of the human MT-IA gene (47), which, despite the general requirement of two or more MREs for metal induction (49, 52, 53), has only one site identical to the above-prescribed core sequence. The conservation of MRE sequences among vertebrates and such invertebrates as drosophila (37, 44) suggests that the characteristics of their *trans*-acting regulatory factors might also be conserved; in contrast, the large divergence between metazoan and yeast MRE sequences (56) suggests considerable differences in their *trans*-acting factors. When a drosophila test system was used to define a metal-responsive promoter region in the drosophila MTn gene (44), the presence of putative MREs with cores both divergent from and identical to the prescribed mammalian sequence obviated the conclusion that the drosophila *trans*-acting factors utilized only those MREs that are optimally active in mammalian test systems. This study resolves this ambiguity by showing that in the sea urchin embryo, another invertebrate, a promoter region containing only those MREs preferred in mammalian systems is active during metal induction.

Among the approximately seven MT genes in the genome of the sea urchin *Strongylocentrotus purpuratus*, at least two (MTA and MTB) are expressed in the embryo under temporal and tissue-specific regulation (41, 58). Embryos cultured in the absence of added heavy-metal ions synthesize an ectoderm-specific 0.7-kilobase (kb) MTA RNA and an endomesoderm-specific 0.85-kb MTB RNA (58). More than 95% of the inherited maternal MT RNA and >90% of the MT RNA synthesized by these embryos is MTA. This prevalence of MTA mRNA over MTB mRNA has been attributed to the more effective induction of the former by metal ions at trace concentrations in the seawater medium (58). A dependence on metal ions even for this apparently basal activity is further suggested by repression of MT gene transcription in embryos treated with a chelator of heavy metals and derepression through the addition of heavy-metal ions (58; D. G. Wilkinson, P. Harlow, and M. Nemer, unpublished data). The ontogenic regulation of *S. purpuratus* MTA (SpMTA) gene expression involves activation of MTA mRNA accumulation in the early blastula stage. Before this stage, the MT genes are apparently refractory to induction by added heavy-metal ions, and no accumulation of MT mRNA is detectable above the preexisting maternal MT mRNA level (41; Wilkinson et al., unpublished data). In this study, by using the microinjection of reporter gene fusion constructs, we focused on the metal regulation of the SpMTA gene and identified the elements in its promoter responsible for metal induction.

MATERIALS AND METHODS

Embryos and embryo RNA. *S. purpuratus* embryos were cultured in synthetic seawater (SSW), and total RNA was extracted (41) from embryos or tissue fractions (38).

Genomic DNA library construction, screening, and sequencing. DNA prepared from the sperm of one sea urchin (35) was the source of 14- to 23-kb *Sau*3A fragments inserted into λEMBL3 (Vector Cloning Systems). An unamplified library of 1.1×10^6 plaques yielded 65 clones that hybridized with a probe consisting of the entire coding region of the

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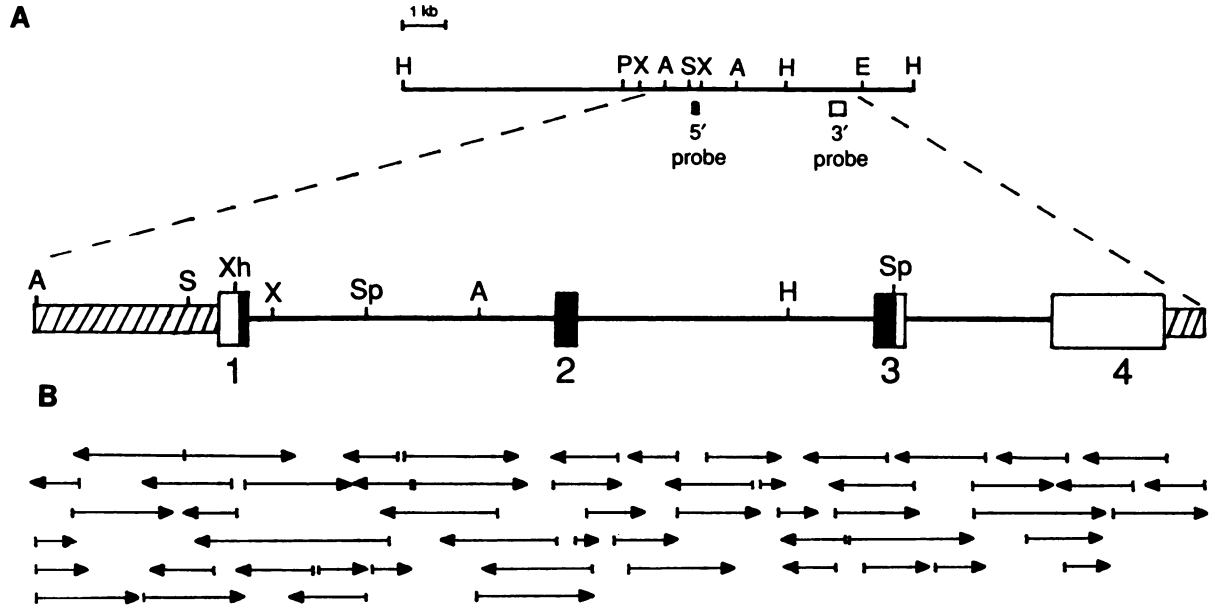

FIG. 1. General organization of the SpMTA gene and sequencing strategy. Locations of exons (1, 2, 3, and 4) are indicated. Symbols: ■, coding regions; □, noncoding regions; ——, intron; ▨, flanking sequence. (A) Partial restriction map of the two λMT206 *Hin*dIII fragments that hybridized to 5′ and 3′ probes. (B) Sequencing strategy. Arrows indicate the direction, location, and extent of overlapping sequenced segments. Restriction endonuclease sites: A, *Acc*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sac*I; Sp, *Sph*I; X, *Xba*I; Xh, *Xho*I.

SpMTA cDNA (42) and 2 that hybridized with a specific 3′ MTA probe (58). Of these two, the clone λMT206 was chosen for restriction mapping and sequencing. Two *Hin*dIII fragments, 9 and 3 kb (Fig. 1), were distinguished by their exclusive hybridization to 5′-coding and 3′-specific MTA probes, respectively (58). Subclones of these fragments were used to prepare progressive deletions for sequencing (58), using a modified chain termination procedure. Sequenced portions were used to specify oligonucleotides, which were end labeled as primers for further sequencing. Nucleotide sequences were analyzed by using VAX computer programs (15).

S1 analysis. A 1.15-kb *Xho*I-*Xho*I fragment containing the proximal 5′-flanking region (see Fig. 3) was end labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase and then digested with *Sph*I to obtain a 0.92-kb *Sph*I-*Xho*I fragment for use in S1 nuclease protection studies (51) with total RNA from either whole embryos, different tissue fractions, or tRNA (40 μg/0.1 pmol of labeled DNA fragment). The S1-protected fragments were analyzed on an 8 M urea–7% acrylamide gel in parallel with sequencing reactions, using an SpMTA subclone and an end-labeled 20-mer oligonucleotide whose 5′ end was complementary to the *Xho*I-cut site.

Construction of SpMT-CAT fusion vectors. We prepared chimeric constructs containing the 5′-flanking region of the SpMTA gene, including its start codon, which was placed in frame with the chloramphenicol acetyltransferase (CAT) start codon in the vector, pUC^{PL}-CAT (5) (obtained from D. Livant, California Institute of Technology). To allow insertion of deletion fragments of the SpMTA gene bearing a common *Sac*I site into the 5′ polylinker region, the *Sac*I site in the 3′ polylinker region of pUC^{PL}-CAT was deleted and a *Sac*I oligonucleotide linker (5′-AGCTTGGAGCTCCA-3′) was inserted at the *Hin*dIII site. This modified pUC^{PL}-CAT was digested with *Sph*I, blunted with T4 DNA polymerase, digested with *Xho*I, and then ligated to a duplex of two complementary oligonucleotides (5′-TCGAGTTCAATTTC ATCACCATGCC-3′ and 5′-GGCATGGTGATGAAATTGA AC-3′). The SpMTA 5′ noncoding region from the *Xho*I site (+59) to the second SpMTA codon (Pro at +83) was thereby inserted into the modified pUC^{PL}-CAT to form intermediate 1. Intermediate 2 was formed from *Sac*I-*Xho*I-digested intermediate 1 by ligation at the *Xho*I site to the 80-base-pair (bp) *Hph*I-*Xho*I fragment (positions −18 to +58) and at the *Sac*I site to a duplex of the two complementary oligonucleotides, 5′-CCCGGGAAAACATATAAAACC-3′ and 5′-GTT TTATATGTTTTCCCGGGAGCT-3′. This inserted duplex contains nucleotides −19 to −34, including the TATA box of the SpMTA gene. The SpMTA-CAT fusion constructs A, B, and C were prepared by ligating 738-bp *Acc*I-*Xho*I, 404-bp *Hha*I-*Xho*I, and 175-bp *Sac*I-*Xho*I fragments, respectively, from appropriate SpMTA subclones to the *Sac*I-*Xho*I-digested intermediate 1. Constructs D and E were prepared by ligating a 760-bp *Hin*dIII-*Sac*I fragment (a 3′ deletion of the *Xba*I-*Xba*I fragment) and a 434-bp *Hin*dIII-*Hha*I fragment, respectively, to the *Hin*dIII-*Sac*I-digested intermediate 2. These constructs were characterized by restriction digestion and sequencing of appropriate regions. All construct DNAs were purified by two rounds of CsCl centrifugation before microinjection.

The a′ and b′ mutant constructs were created by using an oligonucleotide-directed mutagenesis protocol (17), starting with construct A. The oligonucleotides 5′-CATTACAATA TCCTTGGGCAAACAAATAGG-3′ and 5′-GCCTTTCTAG TTTTGATCACGCTTCTATC-3′ were used to change two nucleotides each in MREs a and b, respectively. The a′b′ mutant construct with substitutions in both MREs was generated by inserting the 560-bp *Hin*dIII-*Sac*I fragment obtained from the b′ construct into the *Hin*dIII-*Sac*I-digested a′ vector. The mutations were confirmed by sequencing.

Microinjection of SpMT-CAT fusion constructs into zygotes. The procedure of McMahon et al. (39) was used, with the following modifications. Eggs were dejellied either by

1-min exposure to SSW (pH 5) or by passage through cotton gauze. Microinjection needles were pulled on a Flaming Brown micropipette puller (model P-80/PC) beveled at a 17° angle and siliconized with hexamethyldisilazane (14). One-cell zygotes, maintained at 15°C, were each injected with ~2 pl of a 10% glycerol solution containing 4,000 SpMTA-CAT molecules linearized at the unique *Kpn*I site and a twofold molar excess of 5-kb carrier, *Pst*I-digested *S. purpuratus* genomic DNA. Preliminary experiments showed that the inclusion of carrier DNA augmented CAT activity and that the injection of 4,000 to 12,000 SpMTA-CAT molecules elicited similar CAT enzyme activities. The microinjected zygotes were cultured in situ at 15°C in SSW containing 100 μM ethylenediaminediacetic acid (EDDA). At 19 h postfertilization, blastulae were collected and further incubated for 5 h either in the presence of an effective concentration of 400 μM cadmium or in EDDA. For appreciable expression, 50 to 100 microinjected embryos were assayed for CAT enzyme activity, which for induced embryos was at the low end of the range of values (2 to 80 μU embryo^{-1}) reported for other CAT fusion constructs in this system (16, 18, 33, 55). The embryos were lysed in 250 mM Tris hydrochloride (pH 7.8)–40 mM EDTA–2 mg of bovine serum albumin per ml, using three consecutive freeze-thaw cycles alternating between dry ice-ethanol and 37°C for 5-min intervals; then an equal volume of lysate from uninjected carrier embryos was added.

Assays. CAT enzyme activity was assayed (21) in 150 μl containing 0.2 μCi of [^{14}C]chloramphenicol (49 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) and 1.1 mM acetyl coenzyme A (Sigma Chemical Co., St. Louis, Mo.). Percent acetylation was determined by either direct scintillation counting or imaging with a radioanalytic system (AMBIS; San Diego, Calif.). Enzyme units were calculated by reference to bacterial CAT standards (Sigma). DNA extracted from the embryo lysate (18) was quantified (8), and the numbers of CAT and β-tubulin genes were assayed in each sample by DNA slot blot hybridization with SP6 RNA coding-region probes for either CAT or β-tubulin (24). Standards for gene numbers were slot blots of either SP64-β1-Ref DNA (24) or pUC^{PL}-CAT DNA, the latter added to the same amount of carrier lysate (18) added to the samples. The recovery of DNA was based on 10 β-tubulin genes (24) per haploid genome (0.89 pg of DNA [27]). The average number of CAT genes and their variability at the blastula stage were similar to values determined by others (18, 28).

RESULTS

Structure of the SpMTA gene. The SpMTA genomic clone λMT206 was identified by hybridization with a 3′ MTA cDNA probe shown previously to be specific for a unique MT gene (58). The sequenced region of the SpMTA gene from the *Acc*I site in the 5′-flanking region to 37 bp beyond the 3′ end of the cDNA compared with that of the SpMTA cDNA (Fig. 2) revealed the presence of four exons and three introns (Fig. 1). The 109-bp exon 1 encodes the first 10 1/3 amino acids; the 87-bp exon 2 encodes 29 amino acids, and the 109-bp exon 3 contains a coding region for 24 2/3 amino acids, followed by a noncoding region. A fourth exon, of 416 bp, is unusual in being a completely noncoding 3′ exon. Exons comprise only 20% of this 3,476-bp gene. Whereas the sizes of the first three exons resemble those in other MT genes, the introns of 1,120, 1,085 and 550 bp are considerably larger than other MT introns. Demarcated by the consensus GT(A/G)AG and (C/T)AG splicing signals (6), the introns interrupt amino acid codons 11 (Glu) and 40 (Asn) and divide the 3′ noncoding region. The 52% G+C of the total coding region contrasts with the 63% A+T of the introns, the 67% A+T of the 3′ untranslated region, and the 61% A+T of the 5′-flanking region.

A detailed sequence comparison (Fig. 2) with the MTA cDNA (42) indicates that this genomic clone represents the unique SpMTA gene. The 195 coding nucleotides of this SpMTA gene and the MTA cDNA are 98% identical, and their encoded amino acids are 97% identical. Their divergence in four coding nucleotides alters only two amino acids: Lys for Thr at position 10 and Lys for Val at position 24. Furthermore, the SpMTA gene matches the MTA cDNA in 10 of the 12 codons that distinguish the MTA cDNA from the MTB cDNA (58). The total 3′ noncoding region spanning the last two exons is 91% identical to the corresponding region in MTA cDNA. The principal differences are a 4-bp insertion in the cDNA between positions 3231 and 3232 of the gene and a 29-bp region between 3358 and 3388, which is only 22% identical. From the high degree of polymorphism observed in this sea urchin genome generally (7) and detailed in the case of the Spec1 gene (23), appreciable variation among alleles of sea urchin genes can be expected. The polymorphism of the SpMTA gene is indicated by the heterogeneity of its restriction fragments from different individuals (58) and differences in the restriction maps of the λMT206 SpMTA gene in comparison with partial SpMTA genomic clones containing the SpMTA gene from other libraries (D. G. Wilkinson and M. Nemer, unpublished data). Since the MTA cDNA and the counterpart genomic λMT206 clone were isolated several years apart from different sea urchins, they represent further examples of polymorphic differences tolerated in this species. The SpMTA 3′-specific probe hybridized to the same restriction fragments from λMT206 and the individual genomic DNA used to prepare the library; therefore, the genomic λMT206 clone characterized here contains one of the potential alleles of the SpMTA gene.

Characterization of the transcription initiation site of the SpMTA gene. Three canonical TATA boxes (6), at positions −27, −200 and −424, are 5′ of the translation start site of the SpMTA gene (Fig. 2). To identify the start site of transcription and to determine which TATA box is involved, S1 analysis (51) was performed, using a probe from a region of the gene that included all three TATA boxes (Fig. 3A). Specific SpMTA transcripts were detected as a major band and several closely spaced satellite bands, which were protected from S1 digestion by RNA extracted from pluteus ectoderm (Fig. 3B, lane 2). Heavy-metal induction of this gene was indicated by protection of at least a fourfold-higher amount of the same fragments by ectodermal RNA from plutei incubated with cadmium acetate (Fig. 3, lane 1). Reference to a sequencing ladder revealed that the major band corresponds to a T residue (designated as +1) that is 63 bp 5′ of the *Xho*I site and 22 bp 3′ of the most proximal TATA box. The absence of larger protected fragments for these different RNAs excludes additional start sites for transcription in regions proximal to the other TATA boxes (not shown). The negligible amount of fragments protected by RNA from the mesoendodermal fraction (Fig. 3, lane 3) was consistent with the 10-fold enrichment of MTA mRNA in ectoderm compared with mesoendoderm, as determined by Northern (RNA) blot analysis (58). A variable start site, fluctuating within about three residues of the +1 position, was suggested by the presence of minor satellite bands around the major band in the S1 assays as well as in primer extension studies (not shown). Although variable transcrip-

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-678  GTCTACGTAATTTAGTATGGAAAAATAACCATTTTATCATACAGGTTGATCATCAATATATCATTATGCAGTGAACACCATGAATATTTCGAAATGGAGGGATAGTTGCATTTCTGGCGGAGATATCCGGGTCGGGAA -541
-540  TCATCTACTTTTATTCCCCCTCCCCATTTTTTTCTTCTTCTCCTTGTTCTTCTTCTTTTTCGTTTCTTAATCATGGTATTAATCATTTGCAAATCAATTACATGCCTTTATATATATATAATAATCATGGTGCTACATGA -401
-400  TTTTGAAATACTTTATGGAAACCTTACGTTTTACCAGACCTTGACACAAAGTTGACAGTGTTACCTTTATCATTCACTTCCGTACTGTTTCAGCACATGAGCAACACTTTGTTCTTTGAGGGCAAACGTTTTGGGGGAGG -261
-260  GGAGGGCATCCC[illegible]GGCGCTTTAAAAACAAAAAGGATTTTTGCTTCCCTTCCATATCCCGTATAATTTATCCGACACGAGCCTAGTCATCACCATTCCTGATAGAAGCGTGTGCAAAACTAGAAAGGCCCACCTAGATCG -121
                                                                                                                                      +1
-120  AGCTCCTCAGGCACCGCTAACAGGTTGCCTAGCTCGTGCCTAAACGTGGTGTGTTTACCTATTTGTTTGCACACGGATATTGTAATGAAAACATATAAAACCCCGAATTTCACCAGTTTTTGTCACAAGCTGAAGCGTTT 20

                                                                          MetProAspValLysCysValCysCysLysG
21    GAGTGTGATTTACATTACCTTCTGAAGAAACATACAACTCGAGTTCAATTTCATCACCATGCCTGATGTCAAGTGTGTCTGCTGCAAGGgtaagcaaaaaatcggaatcaatgttctcattaaaataccgagttacagaa 160
                                                                       TCACCATGCCTGATGTCAAGTGTGTCTGCTGCACGG
                                                                          MetProAspValLysCysValCysCysThrG

161   taccgtgtacgtacggtgtagcgtttctagaaacaaaccgctcgatgttacgtgcatacgatgatacgatacagccatggccatgaccatgagaggcccccgatcacgacacacgcagctagctagaccgaattttccgt 300
301   ctctttcgttcgttaagcctcttatttgggtagggtcggctctctctttagagacggaagccgactagacctagggcgcgttcatgcttgattttgaaggcagaatgagcgatttgtaacgctcatcgtaaacgctcatc 440
441   gtaatcgcagttgcacggtgcactgttcatgctcacttctccaggcaattctccttgggtgccttcagctggattcgcaaacctgtgtgcgcatgctacgagagcccatagaggtcagtgcgttgaccccatttaaacgc 580
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1001  cgttcgttatcacagtttttcttcataatcattttcatgtttcttgcccgtttcttcatacttgttacatcaacagttatgtcaataactaagtttttaccaacaagcactgctggatttgtggtttcaaattgcaaga 1140

                                                                                                      luGlyLysGluCysAlaCysPheGlyGlnAspCysCysLysThrGlyGluC
1141  ttgttattactttttaaaatttatatcattatatagactatatatatatatatatatatatattttttttttttaattctgaacacagAGGGCAAGGAGTGTGCCTGCTTTGGACAAGATTGCTGTAAAACTGGTGAAT 1280
                                                                                                      AGGGCAAGGAGTGTGCCTGCTTTGGACAAGATTGCTGTGTAACTGGTGAAT
                                                                                                      luGlyLysGluCysAlaCysPheGlyGlnAspCysCysValThrGlyGluC

      ysCysLysAspGlyThrCysCysGlyIleCysThrA
1281  GCTGCAAGGATGGAACCTGCTGTGGAATATGCACAAgtaagcctttcatcattttcagtgaacccttaaaattaaagcaggcgcgtacgaggatttttaaggggggggggggggggttaacatttataaatcgggcc 1420
      GCTGCAAGGATGGAACCTGCTGTGGAATATGCAAAA
      ysCysLysAspGlyThrCysCysGlyIleCysThrA

1421  gaaaattctgcaacgactcagccgaccgctgaataagtgnngggggggggggggggtagtaagaggacacttttattttttttggtctcgaaattagaaaatttgacatttttgctccgttgggggggggggggggaagg 1560
1561  tcagcttttttaggtcagccatgggaattttttttttaataaaaaaaaaaaacatccagannnngggggggggttttaaggaaatttaggggggggggttacacacaaaaaattaccaaagggtggggtttaaccnn 1700
1701  nnnnnncccctgtgtaagcncctgcatcccagtaattaaggctttttttatactaaactgtttacacaaggcttttaaaagtatttatattcatagaaaaaaattgtcccaattgctcatgcattttttttaatttcttttt 1840
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2261  aaatgaggggcgcagaccaattgtgccctttatatatcctgctgtgtagtctgcatgaaagagaaaatgcagttataccaacaatagaatagagacatttcaatttgatgttattacttggttaattttatttgtccaca 2400

      snAlaAlaCysLysCysAlaAsnGlyCysLysCysGlySerGlyCysSerCysThrGluGlyAsnCysAlaCysEnd
2401  gACGCTGCGTGCAAGTGTGCTAATGGCTGCAAGTGTGGAAGCGGCTGCTCATGCACTGAGGGGAACTGTGCATGCTAGACGAGCTTGGCCCTTTGCTATAGGGATGGCAGgtgagatttcatttattttcatggctacat 2540
       ACGCTGCATGCAAGTGTGCTAATGGCTGCAAGTGTGGAAGCGGCTGCTCATGCACTGAGGGGAACTGTGCATGCTAGACGAGCTAGGCCCTTTGCTATAGGGATGGCAG
       snAlaAlaCysLysCysAlaAsnGlyCysLysCysGlySerGlyCysSerCysThrGluGlyAsnCysAlaCysEnd

2541  agtaaaaattgtttccaggcaaaaacgttagctagttatgggatatgacagggtctgaaatgtaagactgtagatttgcactgcataaagctgtgccatcattttttttaaatagaatttccgagagagcatcaaaat 2680
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2961  ccggaacaaattcattaaccatttctgtgtttcatactttttgtaaaaatatttcaagcatttgttttctctccgtgtctgtttctcttctacagATTGAGCATACAGCTGATGAAGTCACTCAGCGCATCTGCC 3100
                                                                                                  ATTGAGCATACAGCT.ATGAAGTCACTCAGCGCATCTGCC

3101  AACATGCTCCAAGCAGACCTGACTTTGTATATACTTTCGACCCGATGTATTATATTTTTTGTTGTAAGATAGTTGTATATTAAGTTAATTTAATCTTTATTTTTTTCTGAAAATATGAGACTAATTGGCAT....TAATA 3236
      AACATGCTCCAAGCAGACATGACTTTGTATATACTTTCGACCCGATGTATTATATTTTTTGTTGTAAGATAGTTGTA.GTTAAGTTAATTTAAACTTTATTTTTT.CTGAAAATATGAGACTAATTGGCATTATATAGTA

3237  GGACTACTTAAACAACAATTATTTATTAAGGCTGACAGTAATGCCTGATAGAATTTCACTTCATTTTTT.GCTGCCCTGTTTTGTAAATTCTTCAGTCTGTTTCTAACTTCTAT.CAAGTTGTAAATTTGGAATGAAAAT 3374
      GGACTACTTAAACAACAATTATTAATTAAGGCTGACAGTAATGCCTGATAGAATTTTACTTCATTTTTTAGCTGCCCTGTTTTGTAAATTCTTCAGTCTGTTTCTAACTTCTATTCAAGTTGTATTATTATTTCATTACC

3375  AATAATAATAATACATGTGGAGAGTACTTGTAGTGCGCAAACTCCACATGACTAATGTGTTAAAGACTCGCTCATGATTAAATTTAAA.TAATGCTACAAAGTATATTCAGTATGCGTTGCTTAGTTTTTTCTCATGATT 3513
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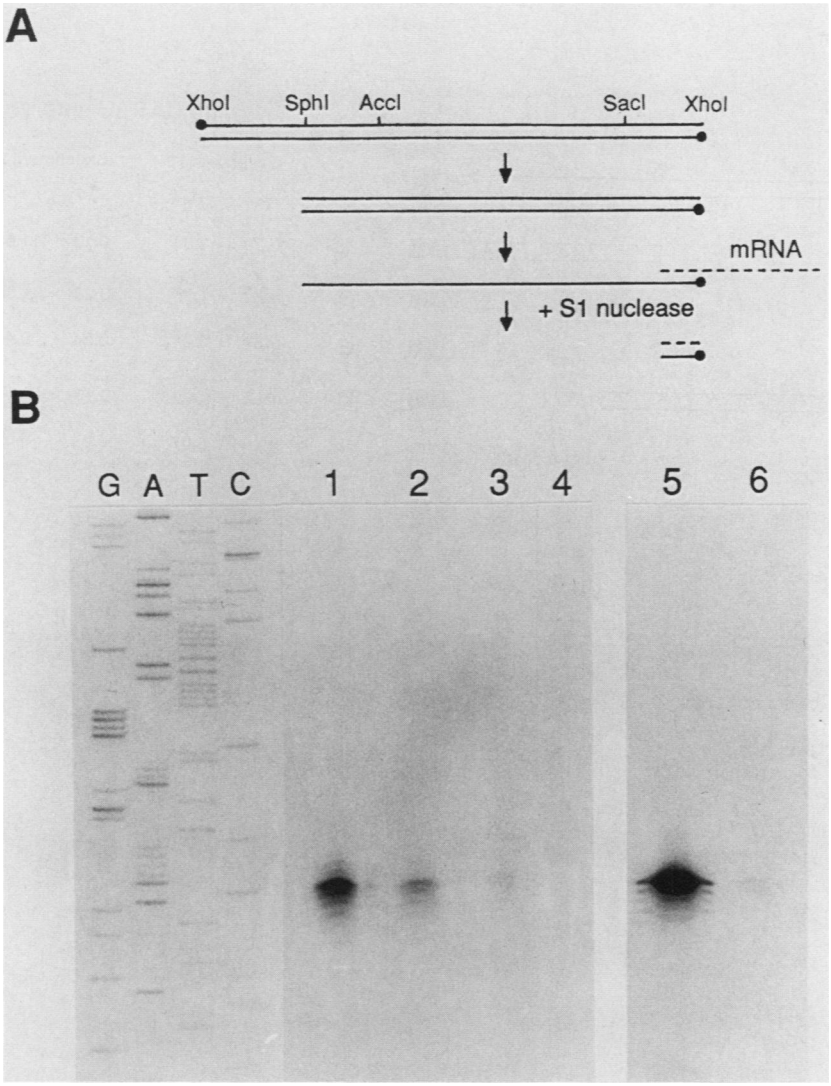

FIG. 3. Mapping of the 5′ end of exon 1 by nuclease S1 protection. (A) Strategy for obtaining an end-labeled (•) *Sph*I-*Xho*I fragment for use in RNA protection against S1 nuclease (see Materials and Methods). (B) S1 protection assay. Sequencing reactions are indicated by G, A, T, and C. DNA fragments protected by various RNAs: total RNA from the ectoderm fraction of plutei, which had been induced by cadmium (lane 1); total RNA from the ectodermal (lane 2) and mesoendodermal (lane 3) tissue of uninduced plutei; yeast tRNA (lane 4); total RNA from 19-h blastulae cultured from fertilization either in normal SSW (lane 5) or in SSW containing 10 μM EDDA (lane 6).

tion start sites have been observed (2, 20, 32), this heterogeneity in these assays may have been due to either RNA degradation or steric hindrance by the mRNA cap structure. Protection by blastula RNA (Fig. 3, lane 5) indicated that the same start site is utilized earlier in development. With RNA from blastulae cultured in EDDA (Fig. 3, lane 6), protection was considerably diminished, consistent with the decline of MT mRNA (58) and the repression of MT gene transcription (Wilkinson et al., unpublished data) under these conditions of heavy-metal ion deprivation.

Identification of the metal-responsive region of the SpMTA gene. Through the mutagenesis of two MREs of the mouse MTI gene, the functional core of mammalian MREs has been shown to be the 8-bp sequence TGCRCNCS (12, 50). In the

FIG. 2. Nucleotide sequence of the SpMTA gene. In the genomic sequence, introns are in lowercase, exon sequences are in boldface uppercase, and flanking sequences are lightface in uppercase. Numbering is for the genomic sequence; the T at +1 represents the nucleotide corresponding to the major S1-protected fragment shown in Fig. 3. Below each exon is the SpMTA cDNA sequence (42), in which a boldface capital indicates the same nucleotide as, and a lightface capital indicates a different nucleotide from, the genomic one. The predicted amino acid sequence of the SpMTA gene is shown above the exons; that of the cDNA is shown below. The TATA boxes are underlined. The putative 5′ MREs (a at −46, b at −146, c at −253, and d at −600) and two intronic MREs are indicated by half arrows below the site to indicate orientation with respect to the direction of transcription; filled and open arrowheads indicate sites with identity and one mismatch, respectively, to the core MRE (TGCRCNCS). In the 3′ noncoding region, dots below the sites indicate an inverted repeat noted by Wilkinson and Nemer (58).

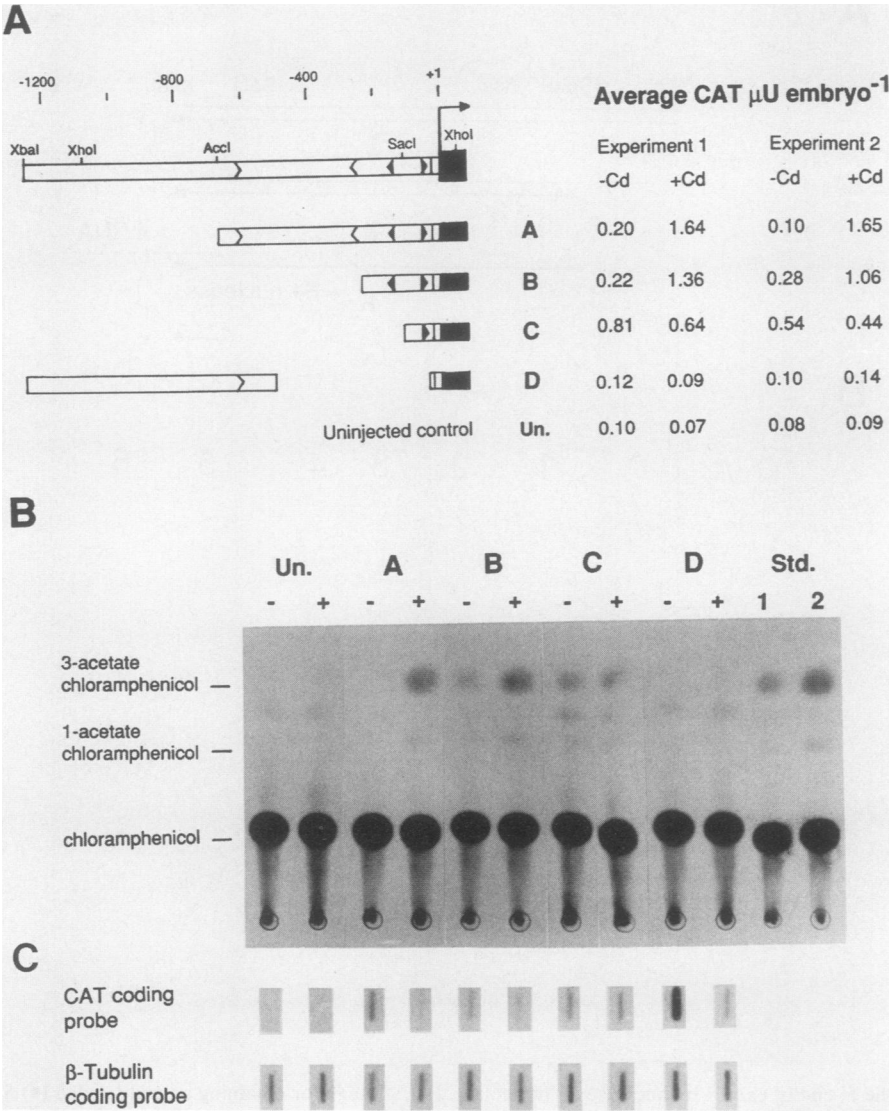

FIG. 4. Identification of the metal-responsive region of the SpMTA gene. (A) Summary of microinjection experiments using SpMTA-CAT fusion constructs. The extent of the 5′-flanking region of the SpMTA gene included in each CAT fusion construct (A, B, C, and D) is diagrammed. Arrowheads indicate the location and orientation of the four putative MREs; the bar indicates the TATA box; +1 marks the start of transcription. CAT values are tabulated for two independent experiments in which the four constructs were injected on a single day, using a single batch of eggs. The uninjected controls (Un.) and injected embryos were induced with cadmium (+Cd) or uninduced (−Cd), as described in Materials and Methods. The average number of CAT genes per injected embryo ranged from 0.6×10^5 to 8.2×10^5 and from 4.8×10^5 to 8.2×10^6 in experiments 1 and 2, respectively. (B) Data from experiment 2, which are representative of the experiments performed. Results of CAT enzyme assays for the uninjected embryos (Un.) and embryos injected with each construct (A, B, C, and D) are shown; positions of 1-acetate chloramphenicol, 3-acetate chloramphenicol, and chloramphenicol are indicated. Bacterial CAT enzyme standards (Std.) were assayed in parallel; standards 1 and 2 were 30 and 100 μU, respectively. (C) Slot blots of each sample DNA probed either with a CAT-coding probe or with a β-tubulin-coding probe.

678-bp 5′-flanking region of the SpMTA gene, we found only two putative MREs identical to this core (MRE a at −53 and MRE b at −146; Fig. 2). Two other putative MREs in this region (MRE c at −253 and MRE d at −607) have a single mismatch relative to the core. To test which SpMTA MREs are functional in heavy-metal induction, we examined the expression of chimeric constructs of the 5′-flanking region of the SpMTA gene with the CAT reporter gene in sea urchin embryos (Fig. 4). Construct A contained all four putative MREs, the 5′-deletion construct B contained only MREs a and b, and C contained only MRE a (Fig. 4). Construct D, containing a TATA box and MRE d together with a still more distal region, tested whether more distal 5′-flanking sequences had any intrinsic metal regulatory regions and whether a spacer similar in size to the 678 bp of the 5′-flanking region in construct A would have an effect on the uninduced level of expression. Construct E (Table 1), containing a TATA box and the region from −244 to −678, tested whether MREs c and d were active independently.

Expression of these constructs was assayed at the mesenchyme blastula stage after injected and uninjected embryos were cultured in EDDA to establish a base line for metal-independent MT gene activity (Fig. 4). Under these conditions, the level of CAT activity in embryos bearing either

TABLE 1. Effects of mutations in MREs a and b

Construct	Sequence[a]		CAT (μU embryo^{-1})[b]	
	MREa	MREb	−Cd	+Cd
A	TGCACACG	TGCACACG	0.08	0.41
a′	TGCcCAaG	TGCACACG	0.07	0.07
b′	TGCACACG	TGatCACG	0.07	0.12
a′b′	TGCcCAaG	TGatCACG	0.05	0.04
E	Deleted	Deleted	0.12	0.11
Uninjected			0.06	0.09

[a] Nucleotide substitutions in the mutant MREs are in lowercase letters.
[b] Values are average activities obtained from two to six experiments involving different batches of embryos. Injected embryos contained on average 1.4×10^4 to 2.9×10^4 CAT genes embryo^{-1} at the blastula stage.

construct A or construct B was not significantly different from the level in the uninjected controls. Therefore, the full promoter region of the SpMTA gene, contained in construct A, had little or no detectable metal-independent activity in embryos cultured in chelator. With construct C, however, a significantly higher uninduced activity was detected. This uninduced activity might be attributed to a positive interference from active vector sequences noted elsewhere (34) and our finding that the uninduced CAT activities of constructs D and E, in which vector sequences are more distant from the start site, is equivalent to that of the uninjected control (Fig. 4; Table 1). Alternatively, if the uninduced expression of construct C is due to the activity of a cryptic basal-level element situated between −120 and the TATA box, then such activity must be repressed by upstream elements in constructs A and B.

Incubation with cadmium resulted in an increase in reporter gene activity in embryos bearing constructs A and B but not constructs C, D, and E. This increase was 1.5 (range, 0.97 to 2.03) and 1.0 (range, 0.70 to 1.19) μU of CAT embryo^{-1} for constructs A and B, respectively. The similarity of induction for these constructs indicated that MREs a and b in construct B are sufficient for metal induction and that MREs c and d are inactive. The lack of induction of constructs D and E confirmed the latter. An analysis with DNA slot blots showed that the injected embryos for each construct contained amplified amounts of CAT DNA. Thus, the lack of expression of constructs D and E could not be attributed to a lack of CAT genes. Construct C, which has only MRE a retained in its normal juxtaposition to the TATA box, showed no metal-induced increase in reporter expression, suggesting a requirement for at least two MREs.

Effects of mutations in MREs a and b. The activities of constructs bearing site-specific changes in MREs a and b were examined to establish the metal regulatory function of these putative MREs in this system and to test whether other, unaltered DNA elements in the 678-bp 5′-flanking region of SpMTA could independently promote transcription of this gene. Specific alterations in either MRE a or b or both drastically decreased their metal inducibility compared with that of the wild-type construct A (Table 1). These results establish the bona fide and sequence-dependent, metal regulatory function of MREs a and b and indicate a requirement for both MREs a and b in the sea urchin metal response. The absence of either uninduced or induced expression in these mutant constructs indicates that basal-level elements are absent in this promoter and that DNA elements within this region that might influence the developmental or cell-type specific expression of this gene must either operate in conjunction with functional MREs or require the activity of elements not present in these constructs.

DISCUSSION

Our analysis of the structure of an invertebrate (sea urchin) MT gene and delineation of its metal-responsive region have indicated features in common with vertebrate MT genes. These features suggest evolutionary conservation of the regulation of MT genes despite some divergence in gene organization.

Characteristics of heavy-metal ion regulation of the SpMTA gene. We have demonstrated that the proximal 240-bp 5′-flanking region of the SpMTA gene is sufficient, and the two MREs in this region are necessary, for heavy-metal induction in sea urchin embryos. MREs a and b, are identical in sequence and position relative to the TATA box to those specified for maximally functional MRE activity in mammalian systems (12, 45, 50). Moreover, the diminution or elimination of the activities of these SpMTA MREs through site-specific mutagenesis implicates them directly and excludes the requirement of other sequences in this region for metal induction. The drastic reduction of activity incurred through mutations in either MRE a or b indicated that neither one is adequate to support substantial activity when the sequence of the other is not conserved. Thus, the requirement for two MREs, retention of the 8-bp core sequence TGCRCNCS, and the position of the proximal MRE within 50 bp of a TATA box may constitute a critical set of conditions for the operation of metal-responsive *trans*-acting factors in both echinoderms and vertebrates. In addition, the natural mismatches in the putative MREs c and d appear to prevent these sites from displaying promoter activity when they alone are juxtaposed to the TATA box in construct E. In mammalian test systems, multiple MREs of greater than two have been shown to amplify the activity obtainable with only two (49, 52). Our results in the sea urchin system suggest that such an amplification is minimal when the additional MREs have defective core sequences. Since the metal-dependent promoter region of the drosophila MTn gene (37) contains nine putative MREs with single mismatches intermingled with two MREs identical to the prescribed mammalian core sequence, it could not be concluded unambiguously that the regulatory factors in this invertebrate recognized the same MRE sequences utilized by vertebrate factors. In the invertebrate system used in this study, we have indeed been able to show that the only two MREs present in a delimited region sufficient for metal induction have sequences identical to the optimal mammalian MRE core sequence and are identifiable as operative, metal-responsive elements by site-specific mutagenesis.

The virtual absence of endogenous MTA mRNA accumulation (Fig. 3) and transcription of MT genes (58; Wilkinson et al., unpublished data) in embryos cultured in chelator indicates that MT gene expression in this system depends largely, if not entirely, on the involvement of heavy-metal ions. This conclusion is supported by the failure to elicit significant metal-independent reporter gene expression from construct A, containing 678 bp of 5′-flanking region. Its metal-independent expression was equivalent to that of the uninjected control embryos or to that of the construct D, which contained 760 bp of more distal 5′-flanking region. The posited dependence of even basal SpMTA gene expression on heavy-metal ions differs from the metal-independent expression noted for several other MT genes, such as mouse MTI (40) and human MTIIA (25, 31). Potentially involved in

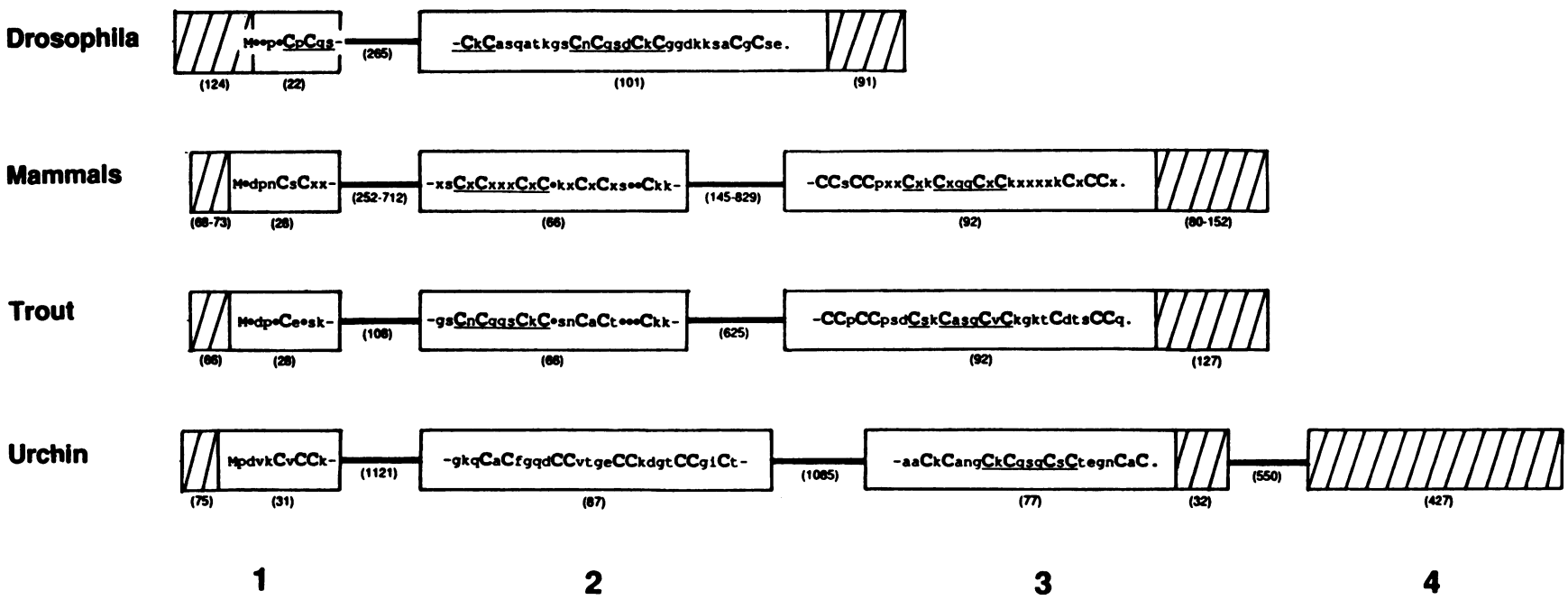

FIG. 5. Comparison of the exon structures of MT genes. Lengths of the coding regions (□) are to scale, whereas lengths of the noncoding regions (▨) and introns (——) are not. Numbers in parentheses indicate lengths (or range of lengths) in base pairs. The amino acid sequence of each exon is shown in single-letter amino acid code; a dash indicates an amino acid interrupted by an intron, and a period indicates a termination codon. The sequences of drosophila (37) and trout (59) are compared with a mammalian consensus (derived from mouse MTI [20] and MTII [48], rat MTI [2], human MTIA [47], MTIB [26], MTI_F [57], and $MTII_A$ [32], and sheep MTIA [46]), where X is a nonconserved amino acid. The spaces needed to align all of the exon 1 sequences and exon 3 of the urchin with exon 2 of the mammals and trout are indicated (●). The regions underlined are the central segments in putative β domains noted by Nemer et al. (42) as well as central segments modified by a single amino acid insertion in the putative α domains of the mammals and trout.

this expression of these other MT genes are such basal regulatory elements as the Sp1-binding sites in their promoter regions. Binding sites for known transcription factors (30), such as Sp1, AP-1 and NF-1, and other previously noted basal elements, such as G boxes (40), were not found in the promoter region of the SpMTA gene. Furthermore, the apparent basal-level activity of the SpMTA gene in embryos cultured in normal seawater was previously indicated as being dependent on trace metals in the seawater (58).

Tissue specificity of SpMTA expression, shown by the exclusive localization of its mRNA in an ectodermal tissue fraction of sea urchin embryos (41, 58), remains unusual for MT genes, even though two human MT genes (26, 57) have also been reported to be expressed tissue or cell type specifically. The expression of SpMTA becomes developmentally restricted to the aboral ectodermal cell lineage (3; M. Nemer, R. D. Thornton, and P. Harlow, unpublished data), in conjunction with a similar cell type expression displayed by at least two other sea urchin genes, the Spec1 (36) and CyIIIa actin genes (11). In the case of the CyIIIa actin gene (9, 13), this specificity has been assigned to the interaction of regulatory factors with *cis* elements in its 5′-flanking region. A comparison of this region of the Sp MTA gene with those of the CyIIIa actin (1) and Spec1 (23) genes revealed the presence of common DNA elements as well as similar patterns of localization and arrangement of these elements. The elements AACCATTT (at −652), ATT ACAT (at −444), and ACCTTTATC (at −338) in the Sp MTA gene are present in the Spec1 gene in the same 5′-to-3′ order and, remarkably, separated by similar intervening distances. Another element, 5′-AGCAAAA-3′ (inverted at −223 in the SpMTA gene) is common to all three genes and, moreover, is located at −200 ± 50 bp from the transcription start site of each. Intriguingly, identical elements were not found in the 5′-flanking regions of the CyI actin (33) or spicule matrix protein (54) gene, both of which are expressed in tissues other than the aboral ectoderm (4, 11). Limitations on the involvement of these common elements in SpMTA promoter activity is suggested by our utilization of constructs in which only MREs a and b were specifically altered. These common elements, although unchanged in their sequences and positions in these constructs, were unable in themselves to support either a basal or an induced activity. This lack of independent activity suggests that these elements play at most a qualifying role in stipulating either the tissue-specific or ontogenic expression of the SpMTA gene.

Structural differences and possible phylogenetic relationships among MT genes. The predicted amino acid sequences of three sea urchin MT cDNAs (10, 42, 58) indicated that the α and β domains of mammalian MTs, which have distinct metal-binding properties (29, 43), have counterparts in the sea urchin MTs but are present in opposite amino and carboxyl halves of these proteins. The mammalian α domain and its counterpart in other organisms contain vicinal cysteines (CysCys pairs), whereas the β domain contains an arrangement of cysteines, CysXCysXXXCysXCys (where X is a noncysteine residue) that occurs so widely as to be posited as the module (the central segment) from which MT proteins have evolved (42). These phylogenetic differences in protein structure may now be pursued by correlating them with structural differences among diverse MT genes. Deuterosome MT genes, represented now by genes in the sea urchin, trout, and mammals, have in common a tripartite arrangement of coding exons and differ from protosome MT genes, which have only two exons, on the basis of the limited example from drosophila (Fig. 5). However, the sea urchin SpMTA gene differs from the vertebrate MT genes first in its structure, by having a fourth, entirely noncoding exon at its 3′ terminus, and second in the arrangement of its encoded protein domains relative to that in vertebrates. The β domain is encoded by exons 1 and 2 in all of 15 mammalian MT genes and in the trout MTB gene. However, the resemblances in cysteine arrangement, presence of the central segment, and size indicate that exon 3 of the sea urchin encodes a β-like domain and corresponds to exon 2 in the vertebrates. Consistent with this apparent switch in positions of exons 2 and 3 is the further observation that the sea urchin exon 2 resembles vertebrate exon 3 in its size and in the presence, but different arrangement, of vicinal cysteines. The distribution of cysteine pairs tends to be skewed toward the carboxyl terminus of the exon in the sea urchin and toward the amino terminus of the exon in the vertebrates. Thus, in each case, their orientation is toward the exon containing the central segment and most of the β domain. Finally, exon 1, which is fairly constant in the size of its coding region and arrangement of cysteines among all MT genes, including the drosophila gene (Fig. 5), appears to take on characteristics that relate it to the protein domain encoded by the adjacent exon 2. Thus, exon 1 adds two cysteines to the seven-cysteine exon 2 to round out the nine-cysteine mammalian β domain; it adds a single cysteine to the seven-cysteine exon 2 of the trout to form an apparent eight-cysteine β domain. In contrast, in the case of the sea urchin, exon 1 adds three cysteines, two of which are vicinal, to exon 2 to complete an apparent α domain.

An ancestral MT gene or its central segment appears to have been duplicated before the divergence of the protosomes and deuterosomes, since the central segments are duplicated in protosomes, represented here by drosophila, and appear to have been duplicated in both mammals and trout, as suggested by the presence of a central segment in exon 2 and a modified central segment, which bears a single amino acid insertion, in exon 3 (both underlined in Fig. 5). In the deuterosomes, these duplicated central segments in two separate exons evolved to produce two domains with vicinal cysteines in one and not the other, independent in their binding of heavy metals (43) and limited in their mutual contact, as observed in the crystal structure of a mammalian MT (19). The considerable differences in the arrangement of vicinal cysteines in exons 2 and 3 in echinoderms and vertebrates, and the potential evolutionary linkage of exons 1 and 2, to encode between them a single type of MT domain, suggest that their switched positions may have resulted from the divergent evolution of their progenitor exons. Although the evolutionary mechanisms are debatable, the varied exonic compositions giving rise to diverse protein structures stand, nevertheless, in contrast to the remarkable conservation in the regulatory apparatus of the MT genes.

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