Structure, Spatial, and Temporal Expression of Two Sea Urchin Metallothionein Genes, SpMTB1 and SpMTA*

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The metallothionein-B genes of the sea urchin Strongylocentrotus purpuratus encode a metallothionein (MT) isoform distinguishable from the MTA isoform. The MTB subfamily consists of at least two genes, MTB1 and MTB2, and possibly two to three others. The unique MTB1 and MTA genes have a high degree of identity but diverge in structural detail and expression. Transcripts of the MTA, MTB1, troponin C Spec 1, and CyIIIa actin genes begin simultaneously to accumulate at an early blastula stage. MTB1 mRNA becomes localized in the embryonic gut and oral ectoderm, whereas MTA, Spec 1, and CyIIIa actin mRNAs are spatially restricted to the aboral ectoderm. Several DNA elements are localized at the same positions in the MTB1 and MTA genes: these include respective CATA and TATA boxes, two metal response elements, and three distinct upstream DNA elements that are also present, and in the same order, in the Spec 1 gene promoter. A heptameric sequence, element A, is present at two sites each in the Spec 1 and CyIIIa actin genes, five sites in MTA, but only one site in MTB1. Most strikingly, the first intron of MTA contains elements not found in the MTB1 introns, including a consensus metal response element, an element A, and the PSA site demonstrated in the CyIIIa actin gene to be linked to the regulation of spatial expression.

Metallothioneins (MTs) are small, cysteine-rich proteins comprised largely of heavy metal-binding domains. Two such domains displaying distinctively characteristic cysteine arrangements occupy the two halves of all vertebrate MTs (1-3), and are oppositely situated in the halves of echinoderm MTs (4), apparently due to the evolutionarily switched positions of their encoding exons (5). A multiplicity of MT isoforms have been observed in different organisms, ranging from two in the mouse (6) to an array in humans, that are encoded by 14 MT genes (7, 8). Whereas MT isoforms may differ sufficiently to have potentially distinct metal-binding properties (9), distinct functionalities have actually not been demonstrated.

The ubiquitous expression of certain MT genes, such as the human MTIIA and MTIIC (7, 8, 10) has led to the view that the MTs perform a housekeeping function. Nevertheless, some MT genes appear to be histospecifically restricted in their expression (9, 11-13). In certain cases the restricted expression has been attributed to DNA methylation (9, 11, 14), thus offering a potential mechanism for developmental switching (9). Whereas MT genes are primarily activated through highly conserved metal response elements (MREs) (see Ref. 5), their activities may also be linked to other gene activities through common DNA elements, such as glucocorticoid response elements and G boxes (15), and a variety of sites for transcription factors (16), such as Sp1, AP-1, or NF-1 (8, 17). Such linkages of MT genes might also involve various histospecific sets of genes.

MT gene expression in Strongylocentrotus purpuratus was shown to be developmentally regulated and to differ in tissue fractions: 0.7-kb MTA mRNA was expressed exclusively in a fraction containing the ectodermal tissues, whereas 0.9-kb MTB mRNA was detected in both ectodermal and endodermal fractions (12, 18). While in situ hybridization of a general MT-coding region probe was detected principally in the aboral ectoderm of uninduced embryos (19) whose MT RNA was predominantly MTA, it was not possible until the present study, using a specific MTA probe, to conclude that MTA mRNA expression is restricted to the aboral ectoderm in uninduced embryos and also in embryos induced with heavy metal ions. MTA is thus a member of the histospecific set of several genes that are expressed in this tissue, which includes Spec 1 (20), CyIIIa actin (21), Spec 2 (22), and arylsulfatase (23, 24). Without the in situ utilization of unique MTB1 probes, the contrastingly ubiquitous distribution of 0.9-kb MTB mRNA among tissue fractions (18) could not be further resolved as the expression of a single gene versus the composite expression of a MTB subfamily of genes. We have now isolated a gene designated MTB, that differs from a previously described MTB1 mRNA (18) but encodes the same MTB isoform. We have used this gene to develop a unique MTB1 probe to monitor the spatial expression of its encoded mRNA unambiguously. The structure and sequence of the MTB1 gene was determined and compared extensively with the previously sequenced MTA gene (GenBank accession no. 30608), in order to establish a basis for evaluating their contrasting patterns of expression.
**Experimental Procedures**

Embryos and Embryo RNA—Embryos of *S. purpuratus* were cultured in synthetic seawater (12), and pluteus larvae were either fixed with 1% glutaraldehyde for *in situ* studies (19) or fractionated into ectodermal and endomesodermal tissue fractions (20). Total RNA was extracted from embryos or tissue fractions (12).

Isolation of the Metallothionein-B, Clone from an *S. purpuratus* Genomic DNA Library and Its Sequencing—A genomic library was prepared from the spermatone of one *S. purpuratus* male (5). The unamplified library of 1.1 × 10^9 plaques yielded 65 cDNAs that hybridized with probes consisting of conserved regions of SpMTA cDNA (4). Twelve of these clones hybridized with probes both for the 5′ and 3′ regions of a MTB cDNA (18). One of these, MTA144, was further characterized by restriction mapping and sequencing. A HindIII 2.4-kb fragment hybridized to the 5′-MTB region probe, while a HindIII-PstI 1.6-kb fragment hybridized to the 3′ region probe (Fig. 1). These fragments were subcloned in pUC18 or pUC19 vectors and sequenced by a modified chain termination procedure (18), using primer oligonucleotides, which hybridized to sequences immediately adjacent to the multilinker site in pUC or to previously determined MT genomic sequences. Problems caused by secondary structures of G-rich sequences with T7/DNA polymerase were overcome and sequenced through the addition of single-stranded DNA-binding protein (U. S. Biochemical Corp.) at a concentration of 5.0 pg/1.5 pg of single-stranded DNA in a reaction volume of 4.5 µl (26). Nucleotide sequences were analyzed using VAX computer programs (27).

Probes for Hybridization—Labeled probes were either DNA or antisense RNA, prepared with templates generated by the polymerase chain reaction (PCR) (28), utilizing oligonucleotides that served as upstream (u) and downstream (d) brackets of regions corresponding to the following probe segments: MT coding region probe spanned u = 8–30 to d = 176–190 of essentially the entire coding region of the MTA cDNA (4); 3′-MTB probe spanned u = 2565–2588 to d = 2580–2585 of the 3′-untranslated region (3′-UTR) of the MTA gene (Fig. 2; numbering corresponds to the MTB gene); 3′-MTB probe spanned u = 2713–2733 to d = 2784–2785 of the 3′-UTR of the MTB gene (Fig. 2); 5′-MTB, leader probe spanned u = 2–22 to d = 82–103 of the MTB gene (Fig. 2).

To prepare ^32P-labeled, single-stranded DNA probes, 20 ng of a given PCR-generated segment and 100 pmol of its downstream-bracketing oligonucleotide were incubated with ^32P-dCTP (800 Ci/mmol, Amersham Corp.), or ^35S-labeled probes were maintained in 10–100 mM dithiothreitol. Three mixtures consisted of MTA cDNA (4) together with either Spec 1 (29), or Spec 1 (29), nick-translated to the same specific activity as described above. The distinct 0.7-kb MTA and 1.5-kb Spec 1 or 1.8-kb CyIIIa actin RNA bands in each lane of a developmental Northern blot were quantified by microdensitometry. The number of molecules/embryo of MTA mRNA was calculated from the value for CyIIIa actin mRNA of 0.9 × 10^6 transcripts/20-h blastula, obtained by Lee et al. (37). In turn, the abundance of MTA transcripts was estimated on the basis of previous measurements that placed their level at 10% of the MTA RNA level (18). An estimation of the number of specific MTB transcripts was made from its calculated fraction of the MTB mRNA developmental profile (18). This was determined by hybridization of the specific 5′-MTB probe to RNA slot blots.

In Situ Hybridization with Specific MTA and MTB Probes—Sections of paraffin-embedded, glutaraldehyde-fixed pluteus larvae, which had been incubated for 4 h with or without medium acetate, were treated essentially as described by Anger et al. (19), except as modified by Wilkinson et al. (38), by changing treatment with proteinase K to 20 µg/ml for 10 min at 23 °C. The sections on slides were incubated under siliconized coverslips in 50% formamide, 5 × SSC, 1 × Denhardt, 10% dextran sulfate together with 200 µg/ml at 50 °C for 18 h. Slides were washed extensively in 5 × SSC at 50 °C, in 5 × SSC with 50% formamide at 37 °C, then at the indicated stringency wash prior to treatment with RNase A (20 µg/ml at 37 °C). The 280-nucleotide MTA 3′-UTR probe, labeled with ^3H (4 × 10^6 dpm/µg), was used at 0.4 µg/ml. The stringency wash for the MTA 3′-UTR probe was 0.25 × SSC, 35% formamide at 37 °C. Slides were overlaid with Kodak NTB2 photographic emulsion and exposed for 7 days before developing. A ^3H-labeled, MTB 3′-UTR probe was similarly prepared and utilized under similar conditions. Since MTB, RNA was substantially lower in abundance, the ^32P nucleotide MTB 3′-UTR probe was 0.25 × SSC, 35% formamide at 37 °C. Slides were developed for 1 week.

**51 Nuclease Protection Analysis of RNA**—A 0.95-kb HindIII-XhoI fragment in the proximal 5′-flanking region of MTA144 (Fig. 1) was end-labeled with [γ-^32P]ATP using T4 polynucleotide kinase (U. S.

![Fig. 1. Sequencing strategy. The SpMTB gene, with its 5′ region on the right, is represented in 100-bp segments and shown with several markers: the transcription start site (+1), as ascertained below (Fig. 3), four exons (in boxes), and the restriction sites, HindIII (H), HindII (Hf), PstI (P), SotI (S), SphI (Sp), Xhol (X), and XhoI (Xh). In addition, EcoRI sites were located 1.3 kb further 5′ and 3 kb further 3′. Line lengths are proportional to the extent of sequencing of individual regions and arrowheads indicate their orientation.](image-url)
Biochemical Corp.), then digested with HindIII to produce a 625-bp fragment for S1 nuclease protection analysis (39). Total RNA (40 µg) was hybridized with this denatured, end-labeled DNA fragment, and treated with 50 units of S1 nuclease (Bethesda Research Laboratories) in 300 µl at 24 °C for 30 min. The mixture was analyzed on an 8 M urea, 10% polyacrylamide sequencing gel. Parallel sequencing reactions performed with the HindIII-XhoI DNA fragment employed an end-labeled 20-base oligonucleotide primer whose 5' end was complementary to, and extended one nucleotide beyond, the XhoI cut site. Thus, the fragment ladder was one base ahead of register with the S1-protected fragments.

RESULTS

Structure and Sequence of the MTB1 Gene—The SpMTB1 genomic clone XMT144 was identified by hybridization with a 3' cDNA probe shown previously to detect the 0.9-kb MTB mRNA, but not the 0.7-kb MTA mRNA (18). The sequence (Fig. 2) of MTB1 compared with the previously described MTA and MTB2 cDNAs (4, 18) and the positions of GT/AG intron junctions revealed a 4-exon structure similar to that of the MTA gene (5). The first three exons contain coding regions, while the fourth exon is entirely noncoding. As in MTB2, the introns of MTB1, are larger than those in vertebrate MT genes. The MTB1 introns are 736, 764, and 690 bp, compared to 1,120, 1,085, and 550 bp for MTA.

The nucleotide sequence of SpMTB1. Intron 1 boundaries and exons (in bold, uppercase) were confirmed by alignment with the MTB2 cDNA sequence (18). The transcription start site (+1) was ascertained by S1 nuclease protection assay (Fig. 3). Emphasized DNA elements include a CATA box at -27 to -23, MRE sequence at -144 to -151, as well as elements (-215 to -221), (-624 to -631) (Ref. 5). To indicate regions that might serve as discriminating probes or as potential regulatory regions, we have aligned the MTB1 gene sequence (first row) with 5' flanking and untranslated, as well as 3'-untranslated, sequences of the MTA gene (5) and MTB2 cDNA (18) in the second and third rows, respectively.

FIG. 2. The nucleotide sequence of SpMTB1. Introns (in lowercase letters) were identified by their GT/AG boundaries and exons (in bold, uppercase) were confirmed by alignment with the MTB2 cDNA sequence (18). The transcription start site (+1) was ascertained by S1 nuclease protection assay (Fig. 3). Emphasized DNA elements include a CATA box at -27 to -23, MRE sequence at -624 to -631, and 110-bp 5' leader sequence, 110-bp 5' leader that were not in the transcribed leader of MTB2, but corresponded to 10 nucleotides immediately 5' of its start site (-1 to -10 nucleotides).

[The figure shows a sequence alignment and structural features of the SpMTB1 gene.]
Extended one nucleotide beyond the Hindlll-XhoI DNA fragment and the ladder. The illustrated mRNA sequence is the reverse complement of the actual sequence determined. A 20-base oligonucleotide used in parallel sequencing reactions and 3) or ectodermal complement of the actual sequence determined. That shown previously (18) to be encoded by the gene and the MTB, cDNA are 98% identical, differing by only eight substitutions and three deletions, and contain, wholly within the fourth exon, a 92-bp, highly repetitive nucleotide substitution. The 3'-UTRs (Fig. 2) of the MTB, gene, one from the 3'-UTR of the MTA gene, and the other from the coding region were used to elucidate the expression of mRNAs encoded by members of the MT gene family. The coding region probe, detecting transcripts of all MTB genes, indicated the global distribution of the 0.7-kb MTB and 0.9-kb MTB mRNAs in the pluteus ectodermal and mesoendodermal tissue fractions (Fig. 5A). The ectoderm expressed predominantly 0.7-kb RNA with some 0.9-kb RNA, whereas the mesoendoderm contained predominantly 0.9-kb RNA and only slightly detectable 0.7-kb RNA. The 3'-MTA probe showed that the 0.7-kb MTA mRNA was exclusively ectodermal. The 3'-MTB probe, which represented a multiplicity of, and probably all, MTB-like genes, indicated that

MTB from MTB genes and transcripts. Conversely, probes based on the 5' leader sequences will be marginally effective in distinguishing MTA from MTB but readily distinguish MTB, from MTA and MTB.

Southern blots of genomic DNA from two individuals (a and b) hybridized with a general coding region probe revealed nine and six major bands, respectively, as well as three weakly-detected bands for each (Fig. 4). Considering the generally high degree of polymorphism encountered among sea urchin genes (42, 43) and the possibility of HindIII or EcoRI sites in the regions of the uncharacterized MT genes spanned by the probes, we estimate an upper limit of six S. purpuratus MT genes/haploid genome. An MTA 3'-UTR probe detected a single one of these bands in both individuals, thereby establishing this 3'-MTA probe as an unambiguous detector of the unique MTA gene. A probe consisting of the counterpart 3'-UTR region of MTB (derived from MTB), failed to detect the MTB band, but did detect six and four bands, respectively, in the two individuals, and thus did not distinguish among an apparent MTB-like subclass. The 3' probes thus define a MTB subfamily of genes that comprise the bulk (possibly four members) of the MT gene family. The 4.8-kb band seen in individual a corresponds to the HindIII-EcoRI fragment indicated for MTB, in Fig. 1. A probe from the 5' leader sequence of MTB, detected single restriction fragments not corresponding to bands hybridizing with the 3' probe. In hybridizing to only one band (corresponding to the 2.25-kb HindII fragment in Fig. 1) in the case of the two individuals of Fig. 4, and of two other individuals not shown, this 5' leader probe proved to be an unambiguous detector of the unique MTB gene.

Expression of MTB, and MTA mRNAs in Plateus Tissue Fractions—Two probes derived from the 5' and 3' regions of the MTB gene, one from the 3'-UTR of the MTA gene, and the other from the coding region were used to elucidate the expression of mRNAs encoded by members of the MT gene family. The coding region probe, detecting transcripts of all MT genes, indicated the global distribution of the 0.7-kb MTB and 0.9-kb MTB mRNAs in the pluteus ectodermal and mesoendodermal tissue fractions (Fig. 5A). The ectoderm expressed predominantly 0.7-kb RNA with some 0.9-kb RNA, whereas the mesoendoderm contained predominantly 0.9-kb RNA and only slightly detectable 0.7-kb RNA. The 3'-MTA probe showed that the 0.7-kb MTA mRNA was exclusively ectodermal. The 3'-MTB probe, which represented a multiplicity of, and probably all, MTB-like genes, indicated that

The 5'-flanking region of MTB, contains two 8-bp elements (at −46 and −144, respectively, in Fig. 2) that are identical to two similarly positioned elements in MTA. These DNA elements are demonstrated MREs in MTA (5) and are likely to have similar MRE functionality in MTB. While a TATA box is present at position −27 in MTA, this same position in MTB, is occupied by a CAT box. Such distinctions have been noted among human MT genes; e.g. the CATA in human MTIs (10) compared to the TATA in hMTlb (11) and hMTl (40). However, the rarity of CAT box occurrence is indicated by a survey of 168 eukaryotic genes (41), which showed that 56% contained TATA while only 8% contained CAT. Apparently absent from this 0.7-kb upstream region of both genes are G boxes and binding sites for such transcription factors as Sp1, AP-1, or NF-1, that might support basal level transcription (8, 15-17). Features of the MTB, and MTA genes are further compared under “Discussion.”

The MTA gene (5) is clearly distinguishable in its coding and 3'-UTR sequences from the MTB, gene (Fig. 2) and the MTB, cDNA (18), and in its 5' leader from MTB, but contains the 79-kb leader of MTA as 84% identical to a region of the 5' leader of the MTB, cDNA by virtue of only five substitutions, single deletions of nine and three nucleotides, and two deletions each of one and two nucleotides (Fig. 2). Consequently, probes based on the 3'-UTR sequences will not readily distinguish MTB, from MTB, but can distinguish

FIG. 3. Transcription start site determined by S1 nuclease protection. The assay described under “Experimental Procedures” was performed with total RNA from the mesoendodermal (lanes 1 and 3) or ectodermal (lanes 2 and 4) tissue fractions of control (lanes 1 and 2) or Cd(II)-induced (lanes 3 and 4) pluteus larvae. Lane 5 was tRNA. A 20-base oligonucleotide used in parallel sequencing reactions extended one nucleotide beyond the HindIII-XhoI DNA fragment (Fig. 1) used to generate the S1-protected fragments, thus accounting for the one base out of register between the SI-protected fragments and the ladder. The illustrated mRNA sequence is the reverse complement of the actual sequence determined.

FIG. 4. Gel blots of restriction digests of genomic DNA. Blots of sperm DNA from separate individual adult males (a and b), which were digested with HindIII and EcoRI then electrophoresed, were probed successively with 3'-MTA at high stringency (lanes 1), a coding region probe (lanes 2), 3'-MTB at low stringency (lanes 3), and 5'-MTB, at high stringency (lanes 4). The same gel blot was used for each successive hybridization, except that lane 4 (individual b) was a different blot. Markers are fragments of HindIII-digested λ and HaeIII-digested φX174.
Sea Urchin Metallothionein Gene Structure and Expression

The levels of the different MT mRNAs were measured during the period of development to the 24-h blastula stage, and compared with those of other RNAs, known to begin their accumulation in the 10–12 h early blastula stage (30, 44, 45). Developmental Northern blots were hybridized with mixtures of MT coding region and either Spec 1 (Fig. 6A) or CyIIIa actin (Fig. 6B) probes, to obtain the amount of 0.7-kb MTA mRNA relative to either of these other RNAs at each developmental stage (the 0.9-kb MTB RNA, at 1/6 the MTA level (18), was not significantly detected in these autoradiograms). Accumulation of each of these RNAs began at the 10–12-h blastula stage (Fig. 6C). We estimated the absolute amounts of the MTA and Spec 1 mRNAs from the value of 0.9 × 10⁵ of CyIIIa actin transcripts obtained by Lee et al. (37) for the 20-h blastula. On this basis, the MTA mRNA rose from ~2 × 10⁴ transcripts/egg to ~2 × 10⁵ transcripts/20-h blastula. The MTB, transcripts could be estimated to be at a considerably lower level of abundance: Since the MTB subfamily of transcripts is at 1/60 the MTA level (18), and the respective maternal and embryonic MTB, transcripts are at approximately 5% and 20% of the MTB subfamily transcripts, MTB, mRNA can be estimated at ~10⁴ transcripts/egg and ~10⁵/20-h blastula. These low levels of abundance precluded direct normalization with high abundance RNAs such as Spec 1 and CyIIIa actin. However, the developmental profile of MTB, mRNA was determined by hybridization of the 5'-MTB, probe with a series of RNA slot blots (Fig. 6C) and the approximate amounts estimated in relationship to total MTB (Fig. 5B) and to MTA mRNA (18). It can be concluded that the accumulation of MTB, and MTA mRNAs begins simultaneously with that of the Spec 1 and CyIIIa actin mRNAs. We have also noted (data not shown) that accumulation of the MTB subfamily transcripts, as gauged by the 3'-MTB probe, also begins at the 10–12-h blastula stage, and when embryos are incubated in a high concentration of ZnSO₄ (0.5 mM) from 1-h postfertilization on induction of the accumulation of either MTA or MTB mRNA does not become evident until this same blastula stage. Thus, if the activation of transcription of the MT genes accounts for the developmental

FIG. 5. Expression of transcripts of the MTA and MTB, genes and the MTB subfamily. A, mesodendodermal and ectodermal tissue fractions of pluteus larvae. Total RNA (7 μg/lane) was extracted from the ectoderm (lanes 1, 3, 5, 7, 9) or mesodendodermal (lanes 2, 4, 6, 8) tissue fractions of pluteus larvae, which had been incubated in the absence (lanes 1–8) or the presence of 500 μM cadmium acetate (lanes 9 and 10). Northern blots were hybridized at high stringency with the coding region probe (lanes 1 and 2), 3'-MTA (lanes 3 and 4), 3'-MTB (lanes 5 and 6), or 5'-MTB, (lanes 7–10). The resolved 0.9-kb MTB mRNA and 0.7-kb MTA mRNA have been described previously (18). B, the amount of MTB, mRNA relative to the total MTB-subfamily transcripts. Total RNA (10 μg/lane) from unfertilized eggs (lanes 1 and 2), 6-h cleaving embryos (lanes 3 and 4), 20-h mesenchyme blastulae (lanes 5 and 6), and 60-h pluteus larvae (lanes 7 and 8) was hybridized with 5'-MTB, probe (lanes 1, 3, 5, 7) or an equimolar mixture of 5'-MTB, and 3'-MTB probes (lanes 2, 4, 6, 8). The fraction of MTB, mRNA relative to total MTB-subfamily transcript was estimated from incorporations measured directly in each band, corrected for differences in the number of labeled residues in each probe (e.g. MTB, fraction = [lane 1] - [lane 2 – lane 1]).

FIG. 6. Early developmental profile of MTA and MTB mRNA. From embryos at the indicated stages postfertilization, 5 μg of total RNA on Northern blots were hybridized with a mixture of MT coding region probe and either Spec 1 (A) or CyIIIa actin (B) probe, and 12 μg of total RNA were hybridized in slot blots with 5'-MTB, probe (C). Microdensitometric tracing of each lane was performed to assess the relative amounts of the 0.7-kb MTA mRNA and either the 1.5-kb Spec 1 or the 1.8-kb CyIIIa actin RNA. These amounts were normalized to the profile of the 0.7-kb MTA mRNA and the absolute amounts calculated on the basis of the previously determined level of CyIIIa actin mRNA at 20 h (37), and plotted in panel D: MTA (●), Spec 1 (○), CyIIIa actin (×). The relative amounts of MTB, mRNA were similarly quantified and the absolute amounts (Δ) were estimated and plotted in the inset of panel D.
onset of their transcript accumulation, similar restrictions may apply to the induced and uninduced embryos. In the case of Spec 1, the onset of gene transcription has been detected approximately 2 h prior to the detected accumulation of transcripts (45).

Spatial Expression of MTA and MTB, mRNAs in Pluteus Larvae—The spatial expression of MTA mRNA was not demonstrated directly by previous studies employing in situ hybridization but was inferred from the predominant localization in the aboral ectoderm of a general MT-coding region probe (19), about 90% of which in the total uninduced pluteus tion in the aboral ectoderm of a general MT-coding region demonstrated directly by previous studies employing Larvae—The spatial expression of MTA mRNA was not onset of their transcript accumulation, similar restrictions by the use of the specific 3'-UTR probe, reveals this mRNA transcripts of Spec 1, the onset of gene transcription has been detected to be exclusively in the aboral ectoderm (Fig. 7A). The coding region probe used in the earlier study also could not discern specific MT mRNA localization in embryos induced by heavy metal ions since all MT mRNAs were induced in this case. The MTA 3'-UTR probe, however, now shows that MTA mRNA of the metal-induced pluteus larva is present exclusively in the aboral ectoderm (B). In both the uninduced and induced embryos, the gut and oral ectoderm each accounted for <3% of the silver grains.

The spatial expression of the MTB subfamily of transcripts, assayed by the 3'-MTB probe, was found to be uniform among the tissues of the pluteus larva, either in the uninduced (C) or heavy metal-induced (D) embryos. Considering that this probe hybridized with at least two and possibly several genes of the MTB subfamily, this uniform distribution can be interpreted as a composite of these gene specificities, within which individual MTB genes may or may not have more narrow specificities. Since the 5' leader MTB, probe is specific for a unique MTB gene, this probe can be used to identify specific MTB, transcripts and to assess their specific spatial distribution in the embryo. We found an enhanced localization of MTB, mRNA in the oral ectoderm and embryonic gut in both the uninduced (E) and metal-induced (F) pluteus larvae. The aboral ectoderm accounted for <20 and <10% of background-corrected signal, respectively, in uninduced and induced embryos. A count of nuclei indicated that the ratio of cell numbers in these regions of embryos was considerably less than the 5:1 ratio of autoradiographic silver grains; therefore, an enrichment of MTB, mRNA in the oral ectoderm and gut was evident even on a per cell basis. Moreover, this distribution was very nearly the complement of the spatial distribution of MTA mRNA.

DISCUSSION

Specific Patterns of MTB, and MTA Gene Expression—A total of six or seven MT genes has been indicated in S. purpuratus, with at least four of these being distinct from MTA and comprising an MTB subset on the basis of their hybridization with a 3'-UTR probe. The MTB isoform is encoded by at least two of these, MTB1 and MTB2, distinguishable primarily by differences in their 5' leader sequences. Probes specific for MTA and MTB2, on the basis of their hybridization with unique restriction fragments revealed strikingly different histospecific expression patterns. Whereas previous studies utilizing a general coding region probe were limited to a description of the spatial expression of the entire array of MT mRNAs (19), we have been able to establish with the specific probe that MTA mRNA is restricted to the aboral ectoderm and this same localization holds in embryos induced with heavy metal ions. Therefore, it can also be concluded that induction does not override the restrictions imposed in the uninduced embryo. The spatial pattern of MTB, mRNA expression not only contrasted with the MTA mRNA pattern but was actually its complement, insofar as its expression in uninduced as well as induced embryos was at a high level in both the embryonic gut and oral ectoderm and low in the aboral ectoderm. MTB1 gene expression is either suppressed in the aboral ectoderm or enhanced in both the gut and oral ectoderm. While transcription factors cannot be excluded as specific agents in this regulation, other relationships might be relevant. (i) A posited suppression of MTB1 might be linked to MTA gene activity. Based on the observation that MTA mRNA expression is about 10-fold higher than the expression of the entire MTB mRNA subset in sea water medium, conditions of low metal ion concentration (18), it may be posited that a competition among these genes for MRE-directed transcription factors would favor MTA. Hence, the activity of MTA in the aboral ectoderm would serve to suppress the expression of the MTB genes, whereas the lack of MTA activity in the other tissues would leave MTB expres-

![Fig. 7. Spatial expression of MTA and MTB, mRNAs in the pluteus larva.](image-url) Sections of pluteus larvae, incubated for 4 h either in the absence (A, C, and E) or presence of zinc sulfate (B, D, and F), were hybridized in situ with 3H-labeled 3'-MTA (A and B), 3H-labeled 3'-MTB (C and D), or 32P-labeled 5'-MTB (E and F) probes. Heavy arrows in A and B indicate aboral ectoderm. The aboral ectoderm (a), oral ectoderm (o) and embryonic gut (g) are indicated in E and F. Left and right panels are phase and darkfield photomicrographs, respectively.
sion unimpeded. (ii) Another consideration, favoring the up-regulation of MTB, expression, is the difference between the tissues of the late stage embryo in their cell division status. It has been proposed by Cohen et al. (46) that in the post-gastrula embryo the aboral ectoderm is no longer engaged in cell division, while the cells of the embryonic gut and oral ectoderm are actively dividing. MTB, gene expression may thus be linked to cell division and in this way becomes localized in the actively dividing embryonic gut and oral ectoderm.

Structures, Sequence Elements and Patterns of Expression of the MTB1, and MTA Gene—The MTA and MTB, genes were compared on the basis of percentage identity at 10-bp intervals and interruptions by segments in one having no counterpart in the other (Fig. 8). They have an extensive high degree of identity, including 90% identity in their 0.7-kb 5' flanking regions and coding regions within 3 exons, as well as 85% identity in regions of the second introns and the fourth exons. The former is interrupted by an insert in MTA and the latter by a segment in MTB1, that is highly repetitive in the S. purpuratus genome (18). The most divergent regions are the ~30% identical interiors of the first and third introns. However, neither the consensus MREs nor the extra copies of element A present exclusively in MTA seem entirely missing from MTB1, but appear as vestiges in homologous regions of the gene. Thus, at a position counterpart to that of MREi1, a vestigial MRE sequence TGCAgACc, appears in the MTB1, intron 1 (lower case letters here and in the sequences below indicate the bases different from those in MTA). Similarly, the MTB1 counterpart to MREi2 is GGGGCACa. While we have not ascertained the functionality of either intronic MRE sequence in MTA, the divergence of both MTB1, counterparts from the prescribed TGCPuCXC sequence (47, 48) appears in the MTB1, and the exclusive presence of canonical MRE sequences (MREi1 and MREi2) in the MTA introns ought to be analyzed for their potential contributions to the two-magnitude greater level of MTA compared to MTB1, mRNA expression in the uninduced embryo, as well as to the different cell-type specificities of these MT genes.

The highly identical 0.7-kb upstream promoter sequences contain the elements designated 1, 2, and 3, which are also present in the Spec 1 promoter region in the same order and approximately the same spacing as in MTA and MTB1. While these common features might be related to the concurrent blastula-stage activation of these genes, other DNA elements have already been implicated as being involved in the temporal regulation of the similarly scheduled CyIIIA actin gene expression (52–54). A single 7-bp element, designated "A," is present in MTB1, in contrast to multiples of this element in the other genes: at two sites in the promoters of Spec 1 (Fig. 8) and CyIIIA actin (at positions −171 and −1588 (52)) and at five sites in MTA (Fig. 8). Whereas a multiplicity of element A distinguishes these genes from MTB1, other DNA elements can be expected to be involved in the aboral ecto-

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**Fig. 8.** A diagrammatic representation of the sequence similarities and differences between the MTA and MTB1, genes. The percentage identity of the MTA and MTB1, genes at 10-bp intervals (indicated by the ordinate on the left) is given by a bar graph throughout their lengths from 0.7 kb upstream to the terminus of their last exons. Regions of essentially no identity are presented as triangular inserts on the MTA (above) or MTB1, (below) side. Exons (ex) are boxes (open for untranslated, filled for translated), placed at locations containing their sequences. MREa (TGACACCG) and MREb (CGTTGGCA), and the corresponding TATA and CATA boxes, are shown in both genes. MRE1 (TGACCGAC) and MRE2 (GGGGCGCA) are in MTA introns 1 and 2. Element A is AGCAAAA or TTTTGCCT. Elements 1–3 are ACCCTTA, ATTACAT, and AACCCTTT, respectively. Also shown is the Spec 1 promoter region (43), which contains these elements. Intron 1 of MTA is shown also to contain the P3A-binding site described in the CyIIIA actin gene promoter (48).
derm specificity of these genes, as indicated by the involvement of the distinct elements P3A and P7II in the spatial regulation of CyIIIA actin gene expression (52, 54). At least one of these elements, P3A, is represented perfectly, TGTGTGCAT, at position 526 in the first intron of MTA (5), but is entirely absent from MTB, Fig. 8). The introns of MTA and MTB are thus interestingly different. Introns 1 of MTB and MTA genes cannot, therefore, be limited to their 5'-flanking regions, especially these genes cannot, therefore, be limited to their 5'-flanking regions. Tests of the functionalities of regions of one of these elements, P3A, is represented perfectly, perfect regulation of CyIIIa actin gene expression and to the character of cellular differentiation that their first introns.

In conclusion, the extensive high degree of sequence identity between the MTB and MTA genes can be taken as an indication that they evolved from a common progenitor MT gene, and their subtle differences can be linked to divergences in their expression and their functions in cellular differentiation and to the character of cellular differentiation that evolved with them.

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REFERENCES

1. Hamer, D. H. (1986) Annu. Rev. Biochem. 55, 913-951
2. Furey, W. F., Robbins, A. H., Clancy, L. L., Winge, D. R., Wang, B. C., and Stout, C. D. (1988) Science 231, 704-710
3. Bonham, R., Zafarullah, M., and Gedamu, L. (1988) Mol. Cell. Biol. 5, 267-281
4. Lee, J. J., Shott, R. J., Rose III, S. J., Thomas, T. L., Britten, R. J., and Davidson, E. H. (1984) Nature 309, 110-122
5. Hardin, S. H., Carpenter, D. C., Hardin, P. E., Bruskin, A. M., and Klein, W. H. (1985) J. Mol. Biol. 186, 243-255
6. Shott, R. J., Lee, J. J., Britten, R. J., and Davidson, E. H. (1984) Dev. Biol. 101, 285-306
7. Tomlinson, C. R., and Klein, W. H. (1990) Mol. Reprod. Dev. 29, 329-338
8. Cohen, L. H., Hinegardner, R. T., and McFadden, G. (1981) J. Mol. Biol. 155, 53-65
9. Culotta, V. C., and Hamer, D. H. (1989) Mol. Cell. Biol. 9, 1376-1386
10. Searle, P. F., Stuart, G. W., and Palmiter, R. D. (1987) Experientia Suppl. 52, 407-414
11. Welford, F. C., Devlin, B. H., and Williams, R. S. (1990) Nature 344, 260-262
12. Seifling, E., Lobb, A., Dorsch-Hasler, K., and Schaffner, W. (1985) EMBO J. 4, 3851-3859
13. Westin, G., and Schaffner, W. (1988) EMBO J. 7, 3763-3770
14. These, N., Calzone, F. J., Thiebaut, P., Hili, R. L., Britten, R. J., and Davidson, E. H. (1990) Mol. Reprod. Dev. 25, 110-122
15. Calzone, F. J., Thiebaut, P., Hili, R. L., Britten, R. J., and Davidson, E. H. (1988) Genes & Dev. 2, 1074-1086
16. Davidson, E. H. (1989) Development 105, 421-445