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Metallothionein Genes MTa and MTb Expressed under Distinct Quantitative and Tissue-Specific Regulation in Sea Urchin Embryos

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Sea urchin embryo metallothionein (MT) mRNAs MTa and MTb have distinct cDNA sequences and are transcripts of different genes of a multigene family. These MT mRNAs differ in size and in their 3′-untranslated sequences. They encode proteins that are unusual among MT isotypes in that the relative positions of their cysteine residues are partially out of register, suggesting potential differences in function. In pluteus larvae MTa mRNA is expressed abundantly and exclusively in the ectoderm, while MTb mRNA, which is restricted to the endomesoderm at a low endogenous level, can be induced to a high level by heavy metal ions (M^{2+}). MT mRNA is present in the maternal reservoir of the egg and is predominantly (>95%) MTa mRNA. Endogenous expression in the embryo, which is at a much higher level than in the egg, requires M^{2+} for gene transcription, is developmentally regulated, and is >90% MTa mRNA. When induced by added M^{2+}, however, MTa and MTb mRNAs accumulate to almost equal levels. The differences in the ratios of MTa/MTb expressed endogenously and inductively are not attributable to differences in the stabilities of these MT mRNAs, which were observed under conditions of M^{2+} depletion, or in their inducibilities, which were observed at moderate to high M^{2+} levels. We found, instead, that the MTa gene responds to M^{2+} at a lower threshold level than MTb, so that at very low M^{2+} concentrations the ratio of induced MTa/MTb mRNA is high and equivalent to the endogenous ratio. Thus, endogenous expression of the MTa gene is selectively enhanced in the ectoderm by determinants that are responsive at low M^{2+} threshold concentrations.

Metallothioneins (MTs) are small, cysteine-rich proteins that are induced by heavy metal ions and encoded by one to several genes in the many organisms known to contain them. These proteins bind heavy metals, and it is believed that they are involved in various aspects of heavy metal metabolism, including the detoxification, storage, and intracellular transfer of these ions. The pattern of regulation of multiple MT genes is apparently diverse among higher organisms. In the mouse, for example, the MTI and MTII genes are coordinately induced by heavy metal ions, by glucocorticoids (36), and during embryonic development (1). On the other hand, the human MTIa, MTIIa, and MTIf genes are differentially regulated by various inducing agents (33) and, furthermore, display cell-type-specific expression (34, 41). This diversity is consistent with the human MT genes having distinct physiological functions (33).

Recent observations on the regulated expression of MT mRNA in the sea urchin embryo offer potential insights into the physiological, and especially the developmental, function of these proteins and possibly also into MT gene diversity in a system that is highly amenable to experimentation (28). Nemer et al. (28) found that in the sea urchin endogenous MT mRNA accumulation is temporally regulated during embryogenesis and is largely restricted to ectodermal tissue in the pluteus larva. Moreover, although endogenous MT expression is at a relatively low level in endomesodermal tissues, substantial accumulation of MT mRNA could be induced in this tissue fraction by treatment with Zn^{2+} ions (28). Thus, it seemed possible that the primarily endogenous expression of MT mRNA in the ectoderm and the primarily inductive expression in the endomesoderm might reflect distinct tissue-specific roles in the sea urchin embryo. To examine whether these modes of expression are aspects of the differential regulation of an MT gene family with potentially diverse functionality in this organism, we set out to analyze (i) whether the tissue-specific differences in MT mRNA regulation reflect fundamental differences between endogenous and induced MT gene expression, (ii) whether the MT mRNAs expressed endogenously in the ectoderm are, indeed, different from those induced in the endomesoderm by heavy metal ions, and (iii) if (i) and (ii) are true, whether the MT mRNAs are encoded by different genes.

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MATERIALS AND METHODS

Embryos. Conditions for the culture of *Strongylocentrotus purpuratus* embryos in synthetic seawater have been described previously (28). In certain experiments embryos were cultured in this seawater medium, containing 500 μM ethylenediamine-*N*,*N*′-diacetic acid (EDDA; Aldrich Chemical Co., Inc., Milwaukee, Wis.) or in 500 μM $ZnSO_4$ (Sigma Chemical Co., St. Louis, Mo.), each of which was added 30 min postfertilization. In some experiments embryos were incubated with the indicated concentrations of zinc sulfate (Sigma) or cadmium acetate (J. T. Baker Chemical Co., Phillipsburg, N.J.) for the indicated times. The procedure of McClay and Chambers (23) was used for the preparation of ectodermal and endomesodermal tissue fractions from plutei. Total RNA was extracted from embryos or tissue fractions as described previously (28).

Bacteria and plasmids. The MTa cDNA, which has been sequenced previously (30), was originally obtained as a construct in pBR322 and designated g11 (37). Clones of recombinant pUC plasmids (42) were obtained by standard procedure (21) using JM83 cells rendered competent by treatment with calcium chloride (26). Plasmids were isolated by the alkali lysis method (4), and large-scale preparations

were purified further by equilibrium cesium chloride density gradient centrifugation.

Construction of cDNA library. A cDNA library of poly(A)$^+$ RNA from zinc-induced gastrulae was constructed essentially as described by Maniatis et al. (21), except that second-strand synthesis was accomplished by adding an oligonucleotide tail to the first strand and by using a complementary oligonucleotide as a primer. Briefly, 50 μg of poly(A)$^+$ RNA was annealed with oligo(dT)$_{12-18}$, and first-strand synthesis was achieved with avian myeloblastosis virus reverse transcriptase. Following the removal of RNA by alkali treatment, the cDNA was 3'-tailed with ~10 deoxythymidine residues; and second-strand synthesis was performed with oligo(dA)$_{12-18}$ as a primer for elongation, first with the Klenow fragment of DNA polymerase and then with avian myelobastosis virus reverse transcriptase. The resulting double-stranded molecules were tailed with deoxycytidine residues and then annealed with deoxyribosylguanosine-tailed pUC9 (P-L Biochemicals, Inc., Milwaukee, Wis.) and used to transform competent JM83 cells. Approximately 10^4 transformants per μg of recombinant plasmid were obtained. Clones containing MT sequences were screened with the pMTa (or g11) cDNA.

Sequencing of cDNA. To sequence in both directions, the pMTb insert was excised from the pMTb clone in pUC9 and ligated into pUC19, and then clones were identified that corresponded to each of the two orientations of insertion. Progressive deletions of the insert sequences of these subclones, adjacent to the primer annealing site, were generated either by the method of Henikoff (16) or that of Yanisch-Perron et al. (45). The extent of deletion was determined by the digestion of small-scale plasmid preparations with *Hin*dIII-*Eco*RI followed by agarose gel electrophoresis. A series of clones with deletions at approximately 200-base-pair (bp) intervals were selected for sequencing. The sequencing of these plasmids was achieved by the method of Theurkauf et al. (W. Theurkauf, H. Baum, B. Jeiying, and P. C. Wensink, Proc. Natl. Acad. Sci. USA, in press). Briefly, this involved the generation of a single-stranded template by *Hin*dIII digestion and treatment with exonuclease III, followed by a modified chain-termination procedure (14) with end-labeled M13 15-mer sequence primer (New England BioLabs, Inc., Beverly, Mass.).

Subcloning of MT cDNA sequences into SP65. Specific MT cDNA sequences (see Fig. 1b) were subcloned into the SP65 plasmid (25) for the generation of antisense RNA probes. MT cDNA inserts were excised with *Pst*I and purified before further restriction digestion. Double-digested SP65 was phenol extracted and treated with alkaline phosphatase. Restriction fragments of MT cDNA inserts were purified by agarose gel electrophoresis and electroelution before ligation. The MT-coding region subclone was generated by the digestion of pMTa insert with *Alu*I and ligation of the 210-bp *Alu*I-*Pst*I fragment into *Sma*I-*Pst*I-digested SP65. The MTa 3' probe was prepared by digestion of pMTa insert with *Hha*I, blunt ending of termini with T_4 DNA polymerase (21), and purification of the 320-bp fragment. The 320-bp fragment was digested with *Taq*I, and following phenol-chloroform extraction and ethanol precipitation, the resulting mixture of fragments was ligated into *Acc*I-*Sma*I-digested SP65. Clones were identified that contained the 270-bp *Hha*I-*Taq*I fragment. The MTb 3' subclone consisted of the 130-bp *Taq*I-*Hae*III fragment of pMTb which was purified and then ligated into *Acc*I-*Sma*I-digested SP65. The preparation of SP6 RNA probes was carried out by the instructions of the manufacturer (Promega Biotech, Madison, Wis.).

Genomic DNA blots. Separate sperm DNA preparations were made from individual adults by the procedure described by Lee et al. (18). Fractions of these DNA samples were digested to completion with various restriction enzymes and electrophoresed on 0.7% agarose gels before they were blotted onto nitrocellulose filters by the method of Southern (39). These filters were baked in vacuo at 80°C for 2 h and prehybridized by briefly soaking in 10× Denhardt solution (10)–0.4% sodium dodecyl sulfate (SDS) and by baking for an additional 30 min. Hybridization with radiolabeled probes was achieved overnight at 42°C in 30% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50 mM sodium phosphate (pH 7.0)–5× Denhardt solution–0.1% SDS–0.1% sodium PP$_i$–100 μg of denatured salmon sperm DNA per ml. Low-stringency washes of these filters were in 1× SSC–0.1% SDS–0.1% sodium PP$_i$ at 50°C. High-stringency washes were in 0.3× SSC–0.1% SDS–0.1% sodium PP$_i$ at 68°C.

Analysis of MT mRNA levels. Procedures used for the extraction of RNA from embryos and for electrophoresis and blotting of formaldehyde gels have been described previously (28). Gel blots were prehybridized by briefly soaking in 10× Denhardt solution (10) containing 0.4% SDS and then baking at 80°C in a vacuum oven for 30 min. Hybridization with RNA probes was performed overnight at 42°C in the presence of 50% formamide–5× SSC–50 mM phosphate buffer (pH 6.8)–5× Denhardt solution–0.1% sodium PP$_i$–0.1% SDS–0.5 mg of yeast RNA per ml–0.1 mg of denatured salmon sperm DNA per ml. Final washing conditions at low stringency were 1× SSC, 0.1% sodium PP$_i$, and 0.1% SDS at 50°C. High stringency washes for the specific detection of MTa or MTb RNA were performed in 0.3× SSC–0.1% sodium PP$_i$–0.1% SDS at 74°C for the MTa probe and at 66°C for the MTb probe. Routinely, gel blots were initially hybridized into the MTb probe and autoradiographed, and then the probe was removed from the filter at 90 to 100°C in 0.01% SDS for 10 min. The gel blot was then probed for MTa RNA. The relative levels of MTa or MTb RNA were determined by scanning densitometry by using autoradiograms obtained at different durations of exposure. We ascertained that probe was in excess and that the signal measured densitometrically was directly proportional to the input RNA over the range of amounts of RNA employed in all experiments.

Quantitation of MTa and MTb mRNAs by normalization to a reference RNA. The MTa and MTb RNA bands were usually not well resolved; therefore, to normalize the amounts of MTa and MTb RNA detected on a particular gel blot, the blot was hybridized with the MT-coding region probe and washed at low stringency, and then the relative amounts of MTa RNA and MTb RNA were determined by scanning densitometry of lanes in which these RNAs were clearly resolved (such as the lanes in Fig. 1). Because the ratio of MTa and MTb RNA was thereby established for a given RNA preparation, the inclusion of this RNA on a gel blot in a reference lane allowed the precise calculation of the relative amounts of MTa and MTb RNA in any given sample by hybridizing alternatively with the specific pMTa and pMTb probes. The blot was hybridized first with the pMTb probe, and then the signals in the various RNA samples were compared microdensitometrically with that in the reference RNA. Next, the MTa probe was used for hybridization either with a different gel blot or with the same gel blot in which the MTb probe had been melted off. Again, the signals in the various RNA samples were compared quantitatively with the signal in the reference RNA. Using the ratio of

MTa/MTb in the reference RNA, we could calculate this ratio within and between samples.

Nuclear run-on transcription. Nuclei were isolated from blastulae, as described by Marzluff and Huang (22). The purified nuclear suspension, containing 2 to 4 μg of DNA, was incubated in 0.6 ml of heparin (100 μg/ml)–90 mM KCl–5 mM magnesium acetate–0.5 mM each of GTP, ATP, and CTP–0.5 μM dithiothreitol–0.5 μM spermidine–2.5 μM [α-^{32}P]UTP (410 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) at 22°C for 30 min. The reaction mixture was adjusted to 1% SDS–3.5 M urea–5 mM Tris hydrochloride (pH 8.0)–0.18 M NaCl–2.5 mM EDTA and extracted with phenol-chloroform (1:1). The nucleic acids were ethanol precipitated and suspended in 22 μl containing 2 mM NaCl, 1 mM dithiothreitol, 8 mM Tris hydrochloride (pH 7.5), and 1.2 mM $MgCl_2$, with 1 U of RNasin (Promega) and 2 U of RNase-free DNase (Promega). The mixture was incubated at 37°C for 1 h and then extracted with phenol-chloroform (1:1), and the aqueous phase was passed through a column (Bio-Gel 1.5 M; Bio-Rad Laboratories, Richmond, Calif.), to remove mononucleotides, and then ethanol precipitated and dissolved in 50 μl of 10 mM Tris hydrochloride (pH 8.0)–5 mM EDTA–0.1% SDS. To compare the relative rate of transcription of MT genes in nuclei from that of the control, Zn^{2+}-treated, or EDDA-treated embryos, equal amounts of radioactivity of the recovered nuclear RNA were separately hybridized to 5 μg of the recombinant plasmid containing the MTa 3′ probe sequences, which were immobilized on nitrocellulose as a slot blot. This hybridization was in 30% formamide–5× SSC–50 mM sodium phosphate (pH 7.0)–5× Denhardt solution–0.1% SDS–0.1% sodium PP_i–0.5 mg of yeast RNA per ml–0.1 mg of denatured salmon sperm DNA per ml in a total volume of 0.5 ml at 37°C for 72 h. Following this, the filters were washed at a final stringency of 0.3× SSC–0.1% SDS–0.1% sodium PP_i at 55°C and subjected to autoradiography. In other experiments the filters were then incubated with RNase A at 20 μg/ml at 25°C for 60 min. The first case was equivalent to low stringency; the second case was equivalent to high stringency.

RESULTS

Distinct MT mRNAs. Nemer et al. (28) showed that MT RNA, detected on gel blots by hybridization with the pMTa cDNA clone (originally designated g11) (30, 37), is regulated in its endogenous expression during sea urchin embryogenesis and is inducible by zinc ions. It could not be determined, however, whether the endogenously and inducibly expressed MT mRNA represented one transcript or distinctly different transcripts. The existence of multiple MT RNA transcripts is now indicated by the detection of two MT mRNA size classes on well-resolved gel blots (Fig. 1a). Whereas only a single 0.70-kilobase (kb) MT RNA was detected in control blastulae (Fig. 1a, lane 1), an additional 0.85-kb RNA was induced on incubation of these embryos with zinc ions (Fig. 1a, lane 2). The pMTa clone used for this detection was isolated from a cDNA library of control blastula RNA (37) and, hence, seemed likely to correspond to the smaller MT mRNA. To characterize the zinc-induced, higher molecular weight MT mRNA, we constructed a cDNA library from the RNA of zinc-induced gastrulae and screened this library with nick-translated pMTa. A distinct clone designated pMTb was isolated. Restriction maps of pMTa and pMTb are shown in Fig. 1b. Comparison of these maps revealed several conserved restriction sites located within 100 bp downstream of the MT protein-coding regions, but little similarity in the distal 3′-untranslated sequences (see the sequences presented previously [30] and Fig. 2). In preliminary studies it was found that pMTa and pMTb inserts cross-hybridized and detected both members of the MT mRNA doublet, even at high stringency, which is consistent with the data shown in Fig. 1a. However, probes corresponding to 3′-untranslated sequences of pMTa and pMTb (Fig. 1b) cross-hybridized at low, but not at high, stringency (data not shown), suggesting that these probes can be used specifically to detect the corresponding MT mRNAs. To test this discrimination, the MTa and MTb 3′ probes were hybridized separately to duplicates of a gel blot of zinc-induced blastula RNA. At low stringency both members of the MT mRNA doublet were detected by the probes (Fig. 1c, lanes 1 and 3). When the blots were washed at high stringency, however, the MTa probe remained annealed only to the smaller MT mRNA, whereas the MTb probe specifically detected the larger MT mRNA (Fig. 1c, lanes 2 and 4). The regulation of these MT mRNAs was examined (below) by hybridizing gel blots first with the MTb probe, removing this probe, and then hybridizing with the MTa probe. On each occasion the blots were washed under high-stringency conditions.

cDNA sequence of the MT mRNA MTb. To deduce the sequence of the MTb cDNA, we generated progressively deleted subclones of the pMTb plasmid and then sequenced them by a modification of the chain termination method (12). Overlapping sections of pMTb were sequenced in both directions (Fig. 2).

An open reading frame in the nucleotide sequence of pMTb potentially encoded a 65-amino-acid, cysteine-rich polypeptide (Fig. 2). The amino acid sequence of this MTb protein is substantially homologous with that deduced for

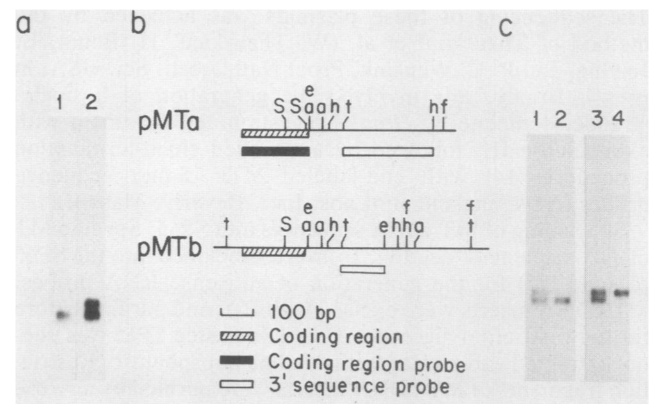

FIG. 1. Resolution and characterization of MT mRNAs. (a) Gel blots on nitrocellulose filters were made after agarose gel electrophoresis; each lane contained 7 μg of total RNA from either control blastulae (lane 1) or blastulae incubated with 500 μM $ZnSO_4$ for 4 h (lane 2). The gel blot was hybridized with the entire pMTa cDNA insert (panel b) which was nick translated to a specific activity of 0.5 × 10^8 to 1 × 10^8 cpm of ^{32}P per μg. (b) Restriction maps of pMTa and pMTb cDNAs were obtained with the enzymes *Sph*I (S), *Alu*I (a), *Hae*III (e), *Hha*I (h), *Hin*fI (f), and *Taq*I (t). The respective regions indicated as 3′ probes were inserted into SP65 to obtain the specific 3′ pMTa and 3′ pMTb probes (see the text). (c) Duplicate gel blots, made as described above for panel a but with only the RNA from Zn^{2+}-treated blastulae in each lane, were hybridized with antisense ^{32}P-labeled RNA transcripts (~1 μg/ml in 5 ml) from the 3′ pMTa probe and washed at low (lane 1) and then at high (lane 2) stringencies; a duplicate blot was probed with 3′ pMTb and was washed at low (lane 3) and high (lane 4) stringencies.

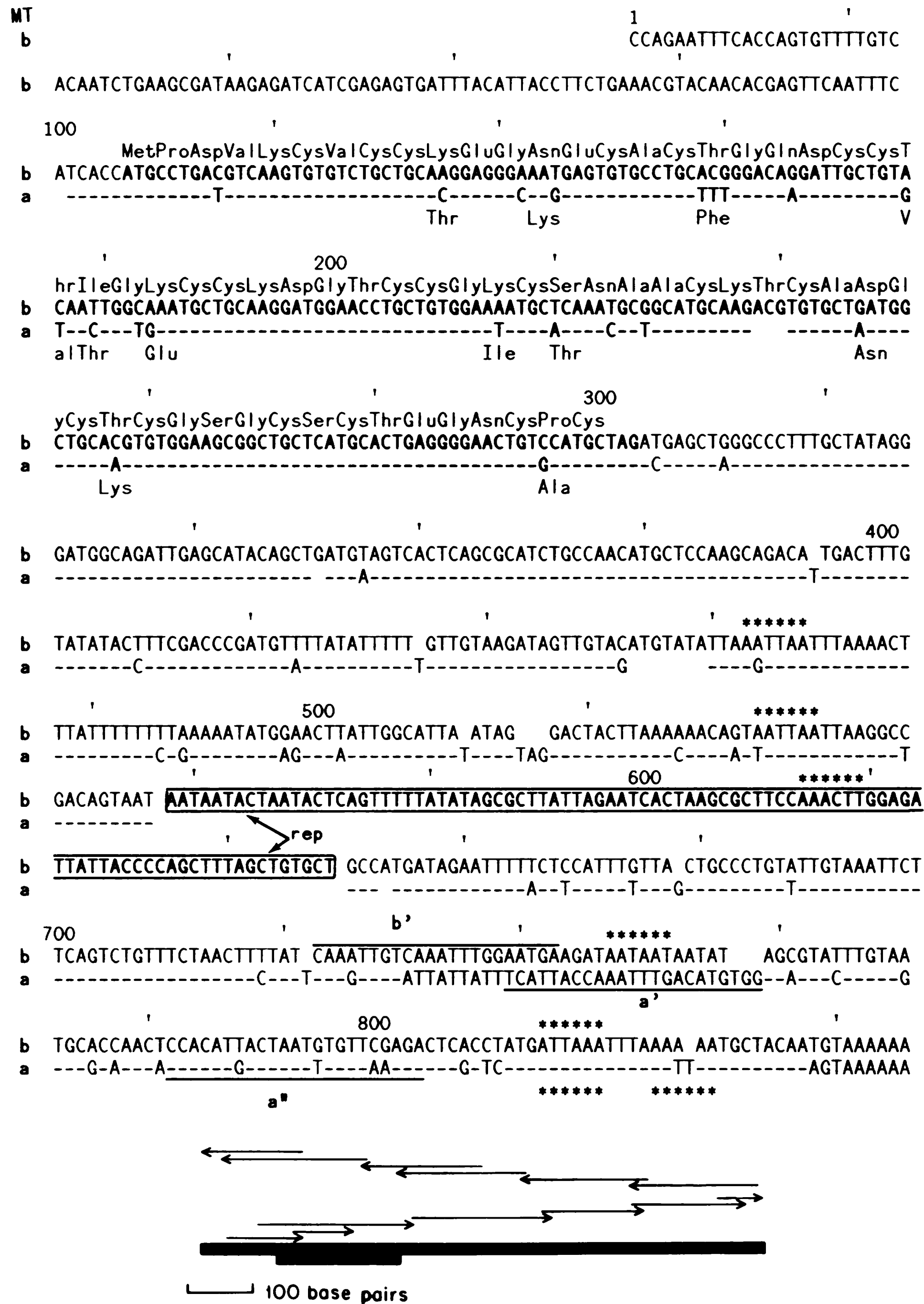

FIG. 2. cDNA sequence of MTb mRNA. The nucleotide sequence of MTb cDNA (b) is shown and compared with that of MTa cDNA (a) (30). Below is presented the sequencing strategy, in which arrows indicate the direction, location, and extent of sequenced, overlapping segments of the pMTb cDNA clone; the thickened part of the horizontal line at the bottom of the figure, representing the pMTb insert, indicates the coding region. Computer-assisted alignment of the nucleotide sequence of MTb (b) shown over that of MTa (a) is numbered from the first nucleotide of MTb that was sequenced. Identical nucleotides are indicated by a dash. The repetitive element, rep at residues 558 to 649 of MTb, is boxed, while the a′ and a″ elements of MTa are underlined and the b′ element of MTb is overlined. Potential poly(A) recognition sites are delineated with asterisks above MTb and below MTa. The predicted MTb amino acid sequence is shown above the MTb nucleotide sequence, and replacements are indicated below the MTa sequence.

MTa (30), with there being 20 cysteine residues in each and a total of 11 amino acid substitutions (Fig. 2). Most strikingly, the single MTa phenylalanine, which is unusual in MTs (8), was replaced with threonine in MTb. We previously identified a sequence, which we called the central segment, in MTa that is completely or partially conserved in all, otherwise diverse, MT proteins (30). The central segment is Cys-X-Cys-X-X-X-Cys-Y-Cys, in which an amino acid with a small R group (e.g., Gly, Ala, Ser, and Thr) is usually in the X or Y position; lysine may also be in the Y

position. MTb also has a typical central segment, differing from that in MTa only by the substitution of a threonine for a lysine residue. An unexpected difference is generated between the MTa and MTb polypeptides by the presence of an additional amino acid residue in the carboxyl-terminal half of MTb, which changes the sequence Cys-Lys-Cys to Cys-Lys-Thr-Cys. Thus, MTa and MTb are unusual among MT isotypes in that, although all cysteine residues are conserved, their relative positions are altered by this additional amino acid.

Computer-assisted alignment of the nucleotide sequences of MTa and MTb mRNAs (Fig. 2) revealed a strong homology (75 to 100%, when assessed at 20-base intervals) throughout most of the length of these RNAs. Two regions within the 3′-untranslated tails of MTa and MTb have interesting differences. One region at residues 722 to 804 consisting of an 85-base segment in MTa contains a 23-base element, designated a′, which is an inverted complement of a downstream element, a″. This arrangement potentially allows the formation of a stable hairpin loop structure in the 3′-untranslated sequence of this mRNA. Such inverted complementary elements are not present in MTb mRNA; however, a segment, b′, is interestingly complementary (19 of 23 bases) to the inverted a′ segment.

Another region in the 3′-untranslated tail that distinguishes these MT mRNAs is a 92-base sequence (rep) in MTb (residues 558 to 649) that is not present in MTa mRNA. It is clear, however, that the sequences on both sides of this 92-base sequence are homologous to sequences within the 3′-untranslated tail of MTa mRNA. When the 92-base segment was hybridized against genomic DNA blots, a large number of bands was detected (Fig. 3). Therefore, at least part of the 92-base element is reiterated in the *S. purpuratus* genome.

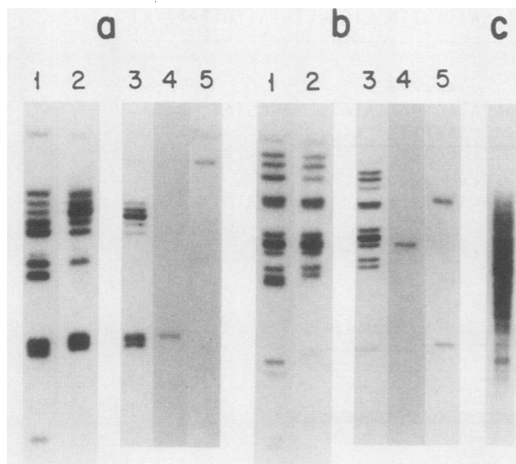

FIG. 3. Gel blots of restriction digests of genomic DNA. The sperm DNA of an individual sea urchin (a) was digested with *Hin*dIII-*Eco*RI, and the DNA of another (b) was digested with *Hin*dIII. Lanes 1 and 2 represent one gel; lanes 3 through 5 represent another. The coding probe (lanes 1) and 3′ pMTa probe (lanes 2 and 3) were hybridized with gel blots at a low stringency; the 3′ pMTa (lanes 4) and 3′ pMTb (lanes 5) probes were hybridized with gel blots at a high stringency. Sperm DNA from another sea urchin digested with *Hin*dIII-*Eco*RI (c) was hybridized at low stringency with a probe that included most of the MTb_{rep} element that was made from a fragment (residues 412 to 635 shown in Fig. 2) of a nick-translated MTb cDNA clone by cutting with *Taq*I and *Alu*I and by purifying by gel electrophoresis before use. In contrast, under the same hybridization conditions, the 3′ pMTa probe (see above) which contained segments homologous to those surrounding the MTB_{rep} sequence revealed nine discrete bands (data not shown).

Both MT mRNAs have poly(A) recognition elements at the usual distance (approx. 30 nucleotides from the site of polyadenylation); however, MTa mRNA has two additional elements and MTb mRNA has three additional elements that are of diverse composition and location.

A multigene MT family in *S. purpuratus*. DNAs from the sperm of different individual sea urchins were used to assess the number of MT genes (Fig. 3). The coding region probe (Fig. 1) hybridized with 11 bands of one individual's DNA digested with *Hin*dIII and with 12 bands of another individual's DNA digested with *Hin*dIII and *Eco*RI. The number of MT genes based on restriction fragment number was indeterminable to the extent that they may have arisen from parental alleles either identically as a single band or distinguishably as separate bands. Also, polymorphism was demonstrable in the number and lengths of MT gene fragments produced by the digestion of DNAs from different individuals with the same restriction enzyme (unpublished data). Nevertheless, the MTs constitute a multigene family in this species. When the probes derived from the 3′-untranslated sequences of pMTa and pMTb cDNAs were used at high stringency, as they were used for discriminating between MTa and MTb mRNAs, the results were consistent with the existence of single-copy MTa and MTb genes. A single restriction fragment of the MTa gene was detected in the genomic DNA of both individuals. In the case of MTb, one band from an individual sea urchin and two bands from another individual sea urchin were seen, which is again consistent with a single gene, because the two bands in the second case were most likely attributable to alleles. In these individuals, eight and nine bands, respectively, represent restriction fragments containing coding regions from MT genes other than MTa and MTb. Of these, six and seven bands respectively, were not subdivided by restriction sites in introns, because they were identical to bands detected by the 3′ MTa probe under conditions of low stringency. Given that some distinct fragments may not be resolved and that alleles may account for at least some of the bands, a lower limit of three or four may be taken as an estimate for the number of MT genes other than MTa and MTb.

MTa and MTb genes expressed endogenously and through heavy metal ion induction. The ratios of endogenous and metal-induced levels of MTa and MTb mRNAs were measured at each of several stages with their specific probes. By using the data presented in Fig. 4a and the normalization procedure described above, we estimate that the endogenous MTa mRNA levels are >10-fold higher than MTb mRNA at the blastula, gastrula, and pluteus stages (Fig. 4a). However, after zinc induction these mRNAs accumulated to nearly equal levels (Fig. 4a). In the pluteus a variable response was obtained. Either the MTa mRNA was already at a high level and apparently not susceptible to further induction or the endogenous level was relatively low and metal induction could be observed (e.g., the two cases in Fig. 4a).

The maternal reservoir of the egg contains MT mRNA; >95% is MTa mRNA. The signal for this RNA is exaggerated in Fig. 4a by extremely long autoradiographic exposure times, after which MTb mRNA was barely detectable. During the period to the blastula stage there is a severalfold increase in the endogenous level of MT mRNA (28), which the data from this study attribute predominantly to MTa mRNA. Because embryos developed normally, at least to the pluteus stage, in the presence of the chelator EDDA even

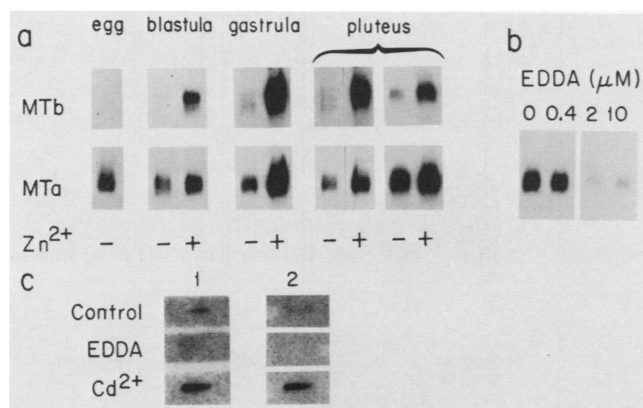

FIG. 4. (a) Relative amounts of MTa and MTb mRNAs expressed endogenously or induced by Zn^{2+} at selected stages. Gel blots were prepared and hybridized with the 3′ pMTb (top panels) and 3′ pMTa (bottom panels) probes successively, as described in the legend to Fig. 1. Total RNA was prepared from embryos, at the indicated stages, that were either untreated (−) or treated for 4 h with 500 μM Zn^{2+} (+). Widely different autoradiographic exposure times were employed for the different stages to clarify the endogenous and induced RNA level in each case. A very long exposure time was used in an attempt to detect MTb RNA in the egg RNA; consequently, a comparable exposure for MTa showed a very high signal that could not be compared quantitatively with the gel blot lanes of the embryonic stages. Several different exposures, together with the procedure for normalization of MTa and MTb signals described in the text, were used for quantitation of these and subsequent data. (b) Effect of development in EDDA on the accumulation of MT mRNA in the blastula. Embryos were developed for 22 h in the indicated concentrations of EDDA to the blastula stage, followed by assay of MT RNA by probing gel blots with the coding region probe (Fig. 1). (c) Transcription rates assayed by nuclear transcript run-on. Nuclei were prepared from 19-h-old blastulae (control), 19-h-old blastulae incubated for an additional 3 h with 50 μM Cd^{2+}, and embryos from the same batch developed to the blastula stage (19 h) in EDDA for use in nuclear transcript run-on labeling with [α-^{32}P]UTP. Equal RNA inputs (5×10^6 cpm of ^{32}P) were used in hybridizations with 5 μg of slot-blotted 3′ pMTa DNA. Lane 1, blots were assayed under stringency conditions designed (Fig. 1) to detect both MTa and MTb transcripts; lane 2, blots were assayed at an effectively high stringency to detect only MTa transcripts.

as high as 500 μM (27), we have used this chelator to study the effect of depletion of heavy metal ions (38) on MT gene expression. We observed the MT mRNA levels in embryos that were developed to the blastula stage in various low concentrations of EDDA to examine quantitatively the metal ion requirements for endogenous MT mRNA expression (Fig. 4b). The result was that >90% of the endogenous expression was eliminated at a chelator concentration between 1 and 2 μM. Because the stoichiometry of binding is approximately 1:1 (38), we can assume that endogenous MTa mRNA expression in the blastula is driven by a heavy metal concentration of <2 μM.

Transcriptional regulation of MT gene expression. We examined whether changes in the rate of transcription are involved in the increased amounts of MT mRNA following heavy metal induction and the decreased amounts resulting from treatment with EDDA (Fig. 4c). Transcript run-on experiments were performed with nuclei from control, Cd^{2+}-treated, and EDDA-treated embryos. These nuclei were allowed to elongate initiated transcripts in the presence of [^{32}P]UTP. Then, equal radioactive inputs of nuclear RNA were hybridized either at low or high stringency with excess DNA containing the MTa 3′-untranslated sequences. At low stringency the transcription of all MT genes was assayed because, according to the gel blots of genomic DNA (Fig. 3), all genes appeared to be accounted for by this probe at low stringency. At high stringency only MTa was assayed. The use of the downstream probe makes it unlikely that the results are attributable significantly to polymerase reinitiation. Furthermore, transcript run-on experiments performed in the presence or absence of heparin gave similar results (data not shown), suggesting that we detected only in vivo-initiated transcripts. Induction by Cd^{2+} stimulated the in vivo transcription rate substantially over endogenous levels, whereas EDDA suppressed the relative rate compared with that of control nuclei (Fig. 4c). Thus, the regulation of MT mRNA levels in Cd^{2+} induction involves enhanced MT gene transcription, and chelator treatment involves suppressed MT gene transcription.

Stabilities of the MTa and MTb mRNAs. We tested the extent to which differences in the stabilities of the MT mRNAs might account for the enhanced endogenous expression of MTa compared with MTb mRNA. MT mRNA synthesis was induced by incubating gastrulae (Fig. 5) with 50 μM cadmium acetate, washing exhaustively, and subsequently incubating in a large excess (500 μM) of EDDA, to deplete the medium and embryos of the heavy metal ions. Because the results of transcript run-on experiments indicated that Cd^{2+} induction increased and incubation in EDDA drastically reduced the amount of transcription, the contribution of new MT mRNA synthesis during the EDDA chase period may not be significant. The effect of the chelator chase on transcription could also be assessed by determining the level of the primary gene transcript. We previously noted that a coding region probe detected an ~3-kb MT RNA in purified nuclei and that the amount of this high-molecular-weight RNA increased substantially on induction with Zn^{2+} (28). Considering the ~3-kb size of the MTa gene (unpublished data), this large RNA most likely represents a primary MT gene transcript. We likewise observed an ~3-kb MT RNA with a specific pMTa probe at time zero in the EDDA chase period (Fig. 5a). In the course of the EDDA chase, the amount of this ~3-kb RNA declined sharply, so that from hour 4 on the amount of this putative primary transcript was <1% of its original value. Therefore, over the subsequent period the reduced level of putative primary transcript indicates that a negligible amount of newly transcribed MT mRNA enters the pool. The doublet of MTa and MTb mRNAs (Fig. 5b) or the separately probed MTa and MTb mRNAs (Fig. 5c and d) was followed during this EDDA chase period. The levels of MTa and MTb mRNA increased for no more than 2 h following the addition of the chelator; then, over the next 20 h mRNA concentrations of both MTs declined with essentially the same decay curve. From the best linear plot of these values on a semilogarithmic scale, their half-lives could be estimated as 2 to 3 h (Fig. 5e). The suppression of MT gene transcription evidenced by the nuclear run-on experiments indicates that the sharp decline in the level of the ~3-kb putative nuclear precursor transcript is due to cessation of synthesis, rather than enhanced decay. In this apparent absence of new synthesis, the decay of these MT mRNAs most likely reflects their half-lives. The stabilities are measured under conditions of heavy metal ion depletion, however, and it has yet to be determined whether or not metal ions influence the stabilities of these mRNAs. In spite of this caveat, the protocol of this experiment may not differ entirely from the events following heavy metal induction. Induction, particularly at a low inducer level, may

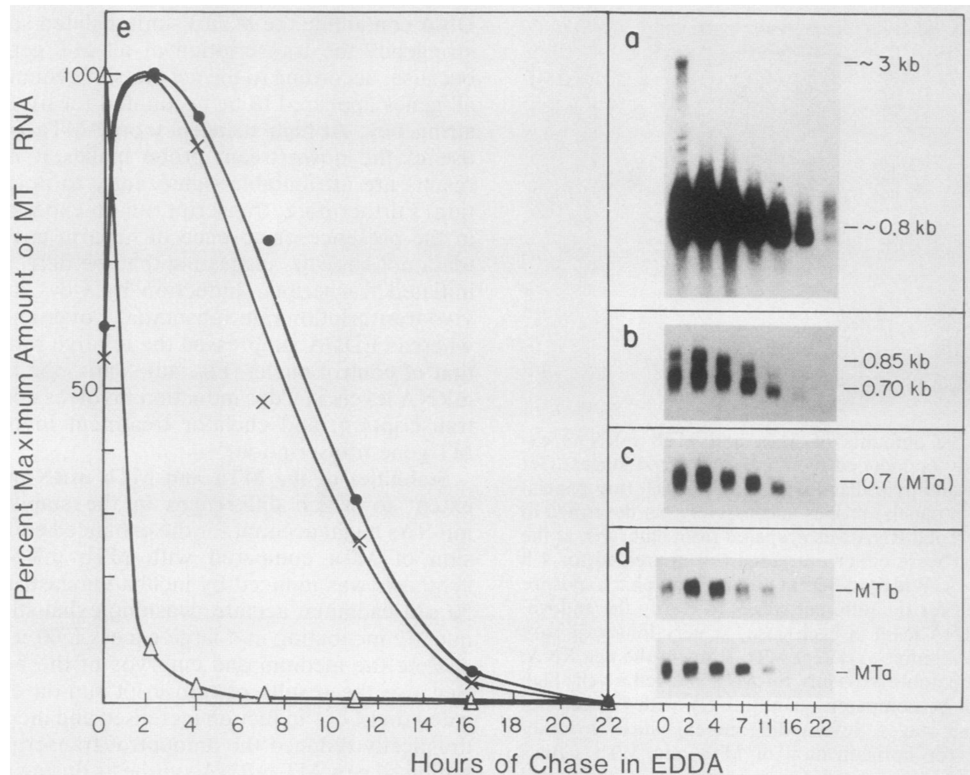

FIG. 5. Decay of Cd-induced MTa and MTb mRNAs. Embryos at the gastrula stage (38 h) were incubated in 50 μM cadmium acetate for 3 h and then washed thoroughly with seawater medium on a nylon screen (Tetko, Inc., Elmsford, N.Y.) prior to suspension in seawater containing 500 μM EDDA, and incubation was continued in the presence of this chelator. RNA was extracted from the embryos and was used for gel blot analysis at the times (in hours) indicated below the lanes. (a) A blot probed with 3′ MTa at low stringency, revealing an ~3-kb MT RNA and the unresolved MT mRNAs. (b) Short exposure of the blot shown in panel a, revealing a resolved MTb-MTa mRNA doublet; microdensitometric tracings of this resolved doublet allowed the quantitation of each. (c) The gel blot shown in panel a washed at high stringency, thus retaining only MTa mRNA, which was quantified densitometrically. (d) Another blot hybridized successively with 3′ pMTb and then 3′ pMTa probes, as described in the legend to Fig. 1. (e) Hybridization signals were measured as described in the legend to Fig. 4 and then plotted relative to the maximum value of each. Symbols: ●, MTa mRNA; ×, MTb mRNA; △, ~3-kb MT RNA.

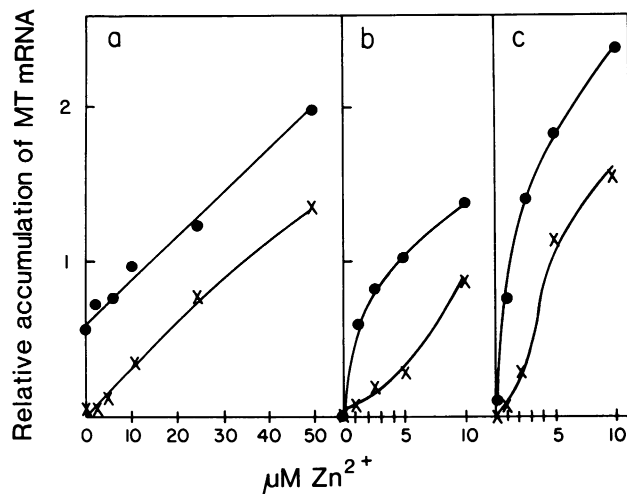

FIG. 6. Responses of MTa and MTb mRNA accumulation to different dosages of Zn^{2+}. Embryos were developed to the gastrula stage in normal seawater medium (a), the gastrula stage in 500 μM EDDA (b), or the pluteus stage in 500 μM EDDA (c). In each case embryos were then suspended in normal seawater medium containing the specified concentration of Zn^{2+}. Total RNA extracted from embryos incubated for 4 h with the indicated $ZnSO_4$ concentrations was used for gel blot analysis, as described in the legend to Fig. 1, by hybridization with pMTb (×) and then pMTa (●) probes. Quantitation was as described in the legend to Fig. 4; a reference RNA of established composition of MTa and MTb mRNAs was used.

result in the buildup of MT protein, which, like the EDDA chelator used in this experiment chelates the inducer and tends to deplete the free heavy metal ion. Thus, decay of MT mRNA at depleted heavy metal levels may not be irrelevant to normal physiological conditions.

Dose responses of the induction of the MTa and MTb genes by Zn^{2+}. The induction of MTa and MTb RNA synthesis was studied as a function of inducer concentration (Fig. 6). In normal gastrulae the amount of MTa mRNA starts at a high level and increases linearly in the range of approximately 0 to 50 μM of added Zn^{2+} (Fig. 6a). In these embryos the increment of additional mRNA per added Zn^{2+} molecule was the same for MTa and MTb mRNA. In other experiments embryos were developed in EDDA to the gastrula (Fig. 6b) and pluteus (Fig. 6c) stage. In these EDDA embryos the endogenous expression of the MT mRNAs was virtually absent; thus, the induced accumulation of MTa and MTb mRNAs starts from a base line near zero. Just as with the metal induction of embryos developed in normal seawater medium, the slopes of mRNA accumulation per added inducer molecule at medium to high concentrations are virtually the same for MTa and MTb mRNA, indicating that the two genes are not different in their inducibility. At the

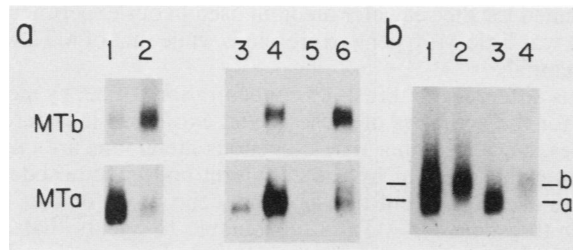

FIG. 7. Tissue distribution of MTa and MTb mRNAs. (a) Total RNA from tissue fractions was probed on gel blots with 3' pMTb (upper panels) and 3' pMTa (lower panels). Normal plutei were used for fractionation (23) into ectoderm (lane 1) and endomesoderm (lane 2). Another batch of plutei was incubated in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of zinc sulfate at 500 μM for 4 h, after which ectoderm (lanes 3 and 4) and endomesoderm (lanes 5 and 6) were prepared. Upper panel gel blots (MTb) were not autoradiographically exposed for the same times as lower panel gel blots (MTa); however, each set of hybridizations was accompanied by hybridization against a reference RNA for quantitation, as described in the legend to Fig. 4. (b) Total RNA from ectoderm (lanes 1 and 3) and endomesoderm (lanes 2 and 4) was prepared from similarly uninduced (lanes 3 and 4) and Zn^{2+}-induced plutei (lanes 1 and 2) and probed with the coding region probe (Fig. 1). Lines are drawn adjacent to the upper band (b = 0.85-kb MTb RNA) and lower band (a = 0.70-kb MTa RNA). This gel blot was quantified directly after different exposures.

lowest concentrations of heavy metal inducer, however, the dose responses of the EDDA-developed embryos show a striking difference between MTa and MTb. In each case the lowest concentration point was obtained by suspending embryos in seawater medium lacking the chelator but containing an additional 1 μM Zn^{2+}. Thus, the total heavy metal to which the EDDA-developed embryos were exposed at this point was <3 μM (as Zn^{2+} equivalent), which includes the estimated value of <2 μM in this medium (Fig. 4b) (25). At this concentration about 10 times more MTa mRNA was induced to accumulate than MTb mRNA. Therefore, below a threshold of <3 μM substantial induction of MTa does occur, whereas that of MTb does not.

Tissue-specific expression of MT mRNAs. To examine the expression of MT mRNAs in tissues of the sea urchin pluteus, ectoderm and endomesoderm tissue fractions were isolated from control and zinc-induced embryos by the method of McClay and Chambers (23). To indicate the limitations due to cross-contamination of the respective tissue fractions, we present results from the fractionation of three different batches of embryos. Specific 3' MTa and 3' MTb probes (Fig. 7a) show that the ectoderm fraction from control plutei contains a high endogenous level of MTa mRNA, with little if any MTb mRNA (Fig. 7a, lane 1) and that the endomesoderm is detectably enriched in MTb mRNA (Fig. 7a, lane 2). A similar result was obtained with a second batch of plutei (Fig. 7a, lanes 3 and 4); however, when these plutei were induced with Zn^{2+}, the ectodermal fraction (Fig. 7a, lane 5) was seen to contain both MTa and MTb mRNAs in substantial amounts while the endomesoderm fraction contained predominantly MTb mRNA, with the level of MTa mRNA being low (Fig. 7a, lane 6) to undetectable (as in Fig. 7b). The relative amounts of endogenous and induced MT mRNAs in these tissue fractions can be evaluated by quantitating these autoradiograms by the normalization procedures described above, i.e., by use of a reference lane containing RNA with a known relative content of MTa and MTb RNA. Alternatively, the quantitative distribution of MT mRNAs between these tissue fractions from Zn^{2+}-induced and normal plutei can be assessed by direct perusal of gel blots after a single hybridization with the coding region probe (Fig. 1) in which the 0.70-kb MTa and 0.85-kb MTb mRNAs were distinguished by their mobilities (Fig. 7b). These two MT mRNA bands were detected at approximately the same levels in the Zn^{2+}-induced ectoderm (Fig. 7b, lane 1). In each of the other cases, however, only a single MT mRNA band was seen. In the endomesoderm the MTb mRNA band in the Zn^{2+}-induced pluteus (Fig. 7b, lane 2) was at a high level compared with a very low level in the normal pluteus (Fig. 7b, lane 4). In contrast to the induced ectoderm, the normal ectodermal fraction contained only the MTa mRNA band (Fig. 7b, lane 3), which was present at a high level. In agreement with the relative MT mRNA levels, which are readily apparent in Fig. 7b, the normalization of the data of Fig. 7a allows the following estimations. (i) Endogenously expressed MTa mRNA is exclusively ectodermal, constitutes >95% of the endogenous MT mRNA in the ectoderm, and is at least 10-fold the endogenous amount of MTb, which is exclusively endomesodermal. (ii) MTa is induced predominantly in the ectoderm, but to a variable extent. Its variable and low level of induced expression in the endomesoderm can be attributed either to a marginal degree of expression in or to a variable and small degree of cross-contamination of this tissue fraction. (iii) The endomesoderm expresses only MTb mRNA, endogenously at a very low level and inductively at a high (10 to 20 times) level. (iv) MTb mRNA can be detected at levels approaching that of MTa mRNA in the ectoderm fraction, but only in Zn^{2+}-induced plutei. It is possible (see below) that MTb mRNA is induced in a part of the ectoderm that differs from the ectodermal tissue in which MTa is expressed.

DISCUSSION

The basis for the differential expression of the MTa and MTb genes. The MTa and MTb mRNAs are distinguishable on the basis of size and, furthermore, are encoded by different MT genes, as ascertained by probing with fragments of different cDNA clones. Only the MTa gene is expressed at an appreciably high endogenous level, and its expression is restricted in the pluteus to the ectoderm and, moreover, to the aboral region of this tissue (2). In contrast, expression of the MTb gene is elicited primarily by heavy metal ion induction. The major site of this induction is the endomesoderm tissue fraction, in particular the endoderm or gut, according to results of recent in situ hybridization studies (2). The specific detection of MTb mRNA in induced ectoderm in this study and the in situ detection of MT mRNA in the oral region of the ectoderm by a coding sequence probe (2) suggest that the oral ectoderm may be an additional site of MTb mRNA induction. Mutually exclusive expression has also been indicated for human MT (hMT) genes: $hMT\text{-}I_E$ in derivatives of endoderm and paraxial mesoderm and $hMT\text{-}I_F$ in derivatives of ectoderm and intermediate and lateral mesoderm (34). Aspects of regulation of the sea urchin MT genes, however, include not only restriction to specific cell types but also an apparently differential regulation of endogenous compared with induced expression. At all embryonic stages endogenous expression is predominantly that of MTa mRNA, whereas induction by exogenous metal ions generates nearly equal levels of MTa and MTb mRNAs. We took several approaches to examine the mechanism of this quantitative regulation.

By essentially eliminating MTa expression during development of embryos to the blastula stage in the chelator EDDA, we demonstrated that the high endogenous level, normal to the blastula, depends on metal ions and does not differ from metal-induced expression in this respect. The actual metal ion requirements for endogenous MT mRNA expression were quantified by measuring MT mRNA levels in blastulae developed continuously in low concentrations of EDDA. The result was that >90% of the endogenous expression was eliminated at a <2 μM concentration of the chelator. Because the stoichiometry of binding is approximately 1:1 (38), we assumed that endogenous MTa mRNA expression in the blastula is driven by a heavy metal concentration of <2 μM. The content of <1 μM each of copper and zinc in the synthetic seawater (28) allows the seawater medium to be implicated in the endogenous expression of MTa mRNA but does not exclude implication of heavy metals stored in the embryo.

The proposition that MTa mRNA is substantially more stable than MTb mRNA, and thus accumulates preferentially, seemed especially worth pursuing, because the maternal MT mRNA, which appears to be stored stably in the egg, was almost exclusively MTa. Embryos induced with Cd^{2+} were subsequently incubated in the chelator EDDA to chase the inducer. Results of transcript run-on experiments indicated that Cd^{2+} induction increases the rate of transcription, whereas treatment with EDDA decreases the transcription rate of the MT genes. These results and the disappearance of an induced ~3-kb putative precursor transcript during the EDDA chase indicate that chasing Cd^{2+}-induced embryos in EDDA either reduces or eliminates the contribution of new MT mRNA synthesis to the mRNA pool. Furthermore, this effect seems to be largely selective for MT mRNAs, because total run-on incorporation of labeled nucleotide in the tested nuclei did not differ among normal, Cd^{2+}-induced, or EDDA-treated embryos and, therefore, seems to be preferable to the classical use of inhibitors of transcription, such as actinomycin. The result that the MTa and MTb mRNAs decay with the same half-life (2 to 3 h) indicates that their stabilities do not account for the differences in their endogenous levels.

To test whether the high endogenous expression of the MTa gene might be due to the fact that it is more inducible than the MTb gene, we measured induction at moderate to high doses of added Zn^{2+}. Essentially the same increments of mRNA accumulation per increment of exogenous Zn^{2+} were obtained for MTa and MTb. The similar dose responses indicate that differences in intrinsic inducibilities of these MT genes do not account for the higher endogenous expression of MTa mRNA.

Development in EDDA allowed clear measurement of the responses of these genes at very low inducer concentrations. The dose-response curves for the EDDA-developed embryos revealed a substantial difference in the induction of these genes at very low concentrations. When the Zn^{2+} concentration was raised from essentially zero to approximately 3 μM, the amount of MTa mRNA was raised substantially above its low base line in these embryos, while there was negligible change in the amount of MTb mRNA. Consequently, MTa was induced to a level >10-fold that of MTb mRNA, thus reproducing the normal MTa/MTb endogenous ratio. It appears, then, that the enhanced endogenous expression of MTa mRNA relative to that of MTb mRNA can be attributed to a difference in the threshold level of exogenous heavy metal ion. Below a 3 μM concentration of heavy metal ion, which is about the concentration we estimated for the seawater medium used in our experiments, there was little MTb gene expression, while that of MTa was substantial.

This difference in threshold concentrations of heavy metal ions for the activities of these genes, expressed in different tissues, suggests either that exogenous metal ions are taken up from the medium by the ectoderm and endomesoderm tissues above different threshold concentrations or that the lower threshold for MTa is attributable to agents that enhance this gene expression specifically only over a range of low concentrations of inducer. While differences in uptake of metal ions by epithelium and gut cannot be discounted, the same differences must apply not only in the pluteus and gastrula (Fig. 6) but also in the blastula, which consists only of epithelium (comprising the presumptive germ tissues), yet it displays similar threshold effects (Fig. 4; unpublished data). That the enhancement of its endogenous expression may be related specifically to a regulatory element in the MTa gene is made plausible by the demonstrated properties of the human hMT-II_a gene (13, 35). In addition to metal-responsive elements and glucocorticoid-responsive elements in the 5'-regulatory region of the hMT-II_a gene, so-called basal level elements (BLEs) have been characterized in this region as part of repeat sequences that behave as enhancers (13, 33). From our results such a BLE, if present in the sea urchin gene, could not act independently of heavy metal ions. The studies bearing on the regulation of the endogenous expression of the mammalian MT genes have not been so rigorous as to exclude a requirement for heavy metal ions. Indeed, the apparent heavy metal ion requirement of the hMT-II_a BLE for binding of a cellular factor in competition with the simian virus 40 enhancer indicates, indirectly, that the BLE function and possibly the endogenous expression of the hMT-II_a gene have this requirement (35).

Comparison of MTa and MTb mRNA structures. A small minority of genes (about 30) have either multiple 5' ends or multiple poly(A) sites (19). About half of these genes with complex transcriptional units are either developmentally or tissue-specifically regulated. Both MTa and MTb can be added to this category, because the former has three and the latter has four potential poly(A) sites within their cDNA sequences. In addition, poly(A) sites still further downstream cannot be excluded without examining the sequences of the MTa and MTb genes. It would be valuable to know whether these multiple sites impinge on either the developmental or tissue-specific regulation of these genes. Other features of their 3'-untranslated sequences serve strikingly to distinguish these mRNAs. MTb has a highly repetitive insert of 92 bases, while MTa, which lacks this repetitive element, instead has a pair of 23-base, inversely complementary elements that allow the formation of a stable hairpin loop structure in the 3' region. Although the functions of 3'-untranslated sequences have not been elucidated, alterations in this region can have physiological repercussions (24). It is apparent from our examination of mRNA half-lives that these structural features do not generate differences in metabolic stability. It remains to be seen, however, whether these features are related to tissue-specific regulation of transcription.

Is the novel difference between isotypic MT proteins a basis for functional differences? The MTa and MTb proteins are substantially homologous, displaying a typical degree of divergence among MT isotypes in their 11 substitutions of noncysteine residues. Nevertheless, MTa and MTb are unusually different in that the positions of their 20 cysteines are out of register, which is the result of an amino acid

residue insertion into MTb. The novelty of such a misalignment can be underscored by noting that all of the mammalian MTs (over 15 that have been sequenced) have all 20 cysteines in perfect register (17) and that all 18 cysteines present in crab MTI are in register with those of crab MTII (20). The 11 cysteines of the amino-terminal halves of MTa and MTb (the homologs of the mammalian α domain) are in register; however, MTa and MTb differ in the alignment of the 9 cysteines of their carboxyl-terminal halves (the homologs of the mammalian β domain). The crystal structure (11) and results of nuclear magnetic resonance studies (5, 32, 43, 44) indicate that the mammalian MT is folded into two domains with distinct properties: the α domain binds a cluster of four metal ions and the β domain binds a cluster of three metal ions. This two-cluster arrangement and the observed nonuniform distribution of different metals between the clusters (6, 32, 43, 44) suggest that the two domains have different properties and that MT performs multiple functions. For example, Cd can be detoxified by sequestration in one cluster, while Zn can be homeostatically regulated by storage in the other cluster from which this metal may be donated to apometalloproteins (9, 40). Moreover, through direct protein-protein interaction, metal interchange can take place between MT molecules (31). The dual domain nature of sea urchin MT, drawn by analogy with mammalian MTs, also implies a dual functionality that has yet to be tested. Therefore, if modifications of the cysteine array in these sea urchin β-homolog domains influence their metal-binding properties, then the MTa and MTb isotypes may afford their respective tissues a specific balance of functions bearing on detoxification, essential metal homeostasis, and donation to apometalloproteins. Heguy et al. (15) reported recently that $hMTI_B$ retains the positions of 20 cysteines in register with those of other mammalian MTs; however, an additional cysteine appearing as a substitution potentially alters its metal-binding properties. Furthermore, the tissue specificity of this hMT suggests, as in the case of the sea urchin MTb, that its isotypic variation may be significant in terms of tissue-specific function.

Functional diversity of the MT gene family and developmental regulation of MT gene expression in cell differentiation. The diversity of regulation of the multigene MT family in the sea urchin is evidenced by the differential expression of the MTa and MTb genes. Whereas both of these genes are capable of becoming induced by exogenous heavy metals and may have a role in detoxification through metal ion chelation, the marginal endogenous expression of MTb suggests that this inductive response may be the primary, if not sole, function of the MTb gene.

A clue to the function of the high basal expression of MTa may be the similar tissue-specific expression of the Spec 1 and CyIIIa action genes, which together with MTa are restricted in the pluteus larva to the aboral ectoderm (2, 3). As noted previously (27, 29), the developmental initiation of Spec 1 and CyIIIa actin mRNA accumulation (7, 18) is concurrent with the onset of MT mRNA accumulation at the early blastula stage and, furthermore, that while Zn^{2+} is an inducer of MT mRNA accumulation during this period, the heavy metal ion is at the same time a suppressor of the activation of the other two mRNA expressions. Zn^{2+} treatment at this early time allows cell division to proceed essentially at a normal pace but delays development in such a way that certain gene expressions that are associated with the early stages are enhanced and others that are associated specifically with differentiated tissues are suppressed (27) (unpublished data). The suppression of these latter genes, which include Spec 1 and CyIIIa actin, may be released by the removal of Zn^{2+} through EDDA treatment (27). Whether or not the action of Zn^{2+} is direct or indirect, the intervention of a chelator, either EDDA, which is added by the experimenter, or MT, which forms in response to Zn^{2+} treatment, serves to alleviate the effects of Zn^{2+}. The maintenance in the aboral ectoderm of a high level of chelator in the form of its endogenous MTa protein can be expected to counter the inhibitory effects of the heavy metal, such as the diminution of Spec 1 and CyIIIa actin mRNA levels by Zn^{2+} treatment of pluteus larva (unpublished data). Consequently, we propose that a tissue-specific balance of gene expression may revolve around the dual role of heavy metal ions, as ultimate repressors of genes, such as Spec 1 and CyIIIa actin, and as inducers of the chelating MT.

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