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# Regulation of Metallothionein Gene Transcription

## IDENTIFICATION OF UPSTREAM REGULATORY ELEMENTS AND TRANSCRIPTION FACTORS RESPONSIBLE FOR CELL-SPECIFIC EXPRESSION OF THE METALLOTHIONEIN GENES FROM *CAENORHABDITIS ELEGANS**

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**Metallothioneins are small, cysteine-rich proteins that function in metal detoxification and homeostasis. Metallothionein transcription is controlled by cell-specific factors, as well as developmentally modulated and metal-responsive pathways. By using the nematode *Caenorhabditis elegans* as a model system, the mechanism that controls cell-specific metallothionein transcription *in vivo* was investigated. The inducible expression of the *C. elegans* metallothionein genes, *mtl-1* and *mtl-2*, occurs exclusively in intestinal cells. Sequence comparisons of these genes with other *C. elegans* intestinal cell-specific genes identified multiple repeats of GATA transcription factor-binding sites (*i.e.* GATA elements). *In vivo* deletion and site-directed mutation analyses confirm that one GATA element in *mtl-1* and two in *mtl-2* are required for transcription. Electrophoretic mobility shift assays show that the *C. elegans* GATA transcription factor ELT-2 specifically binds to these elements. Ectopic expression of ELT-2 in non-intestinal cells of *C. elegans* activates *mtl-2* transcription in these cells. Likewise, *mtl-2* is not expressed in nematodes in which *elt-2* has been disrupted. These results indicate that cell-specific transcription of the *C. elegans* metallothionein genes is regulated by the binding of ELT-2 to GATA elements in these promoters. Furthermore, a model is proposed where ELT-2 constitutively activates metallothionein expression; however, a second metal-responsive factor prevents transcription in the absence of metals.**

Metallothioneins (MT)[1] are a family of structurally related, low molecular weight, cysteine-rich proteins (1). The precise physiological role of MT has not been elucidated. However, evolutionary conservation across many phyla suggests that it serves important roles in cell function. Proposed roles include the following: (*a*) participation in maintaining the homeostasis of essential trace metals; (*b*) sequestration of toxic metals, such as cadmium and mercury; (*c*) acting as a reservoir of essential metals that can be donated to other metalloproteins; and (*d*) protection against intracellular oxidative damage (2).

Exposure of cultured cells or whole organisms to transition metals, ionizing radiation, heat-shock, or oxidative stress induces MT transcription (1, 3–5). Metallothioneins typically occur in multigene families. The mammalian MT family consists of four members designated MT-I to MT-IV (6). It has been commented that all organisms express MT or MT-like proteins. However, not all tissues within an organism will express all MT isoforms. Numerous studies indicate that individual MT family members display specific cellular patterns of expression. Typically, MT-I and MT-II genes are coordinately expressed. It has been shown, however, that MT-2A mRNA levels do not increase in response to cadmium exposure in human proximal tubule cells (7). Expression of the human MT-$I_B$, MT-$I_E$, MT-$I_F$, and MT-$I_G$ genes varies in a cell line-specific manner in response to transition metals (8–12). Mouse MT-I is weakly expressed in testes (13). In addition, MT-III is expressed primarily in neurons, although its mRNA is detected in testes and prostate (14–16). MT-IV is exclusively expressed in differentiating stratified squamous epithelia (17).

Metallothioneins from invertebrates also show highly restricted patterns of constitutive or metal-inducible expression. For example, sea urchin *spMTA* and *spMTB* gene expression is limited to aboral ectoderm and embryonic gut and oral ectoderm, respectively (18, 19). The *Drosophila Mtn* and *Mto* genes are primarily expressed in the midgut (20, 21). In addition, inducible transcription of the two MT genes of the nematode *Caenorhabditis elegans* occurs exclusively in the intestinal cells of larval and adult nematodes (22).

Upstream regulatory elements and transcription factors that control metal-inducible MT transcription have been identified in a variety of species (2, 23). Several models have been proposed describing the regulation of metal-inducible MT expression (24–26). However, the mechanism(s) that regulate cell-specific MT expression have not been extensively examined. Cell-specific expression has been explored in the context of DNA methylation, by using hypomethylating agents such as 5-azacytidine. Correlation between hypomethylation and the stimulation of MT gene expression has been observed for rainbow trout, mouse, and human MT genes (8, 11, 27). Differences in the levels of cadmium-inducible transcription between human MT-$I_G$ and MT-$I_F$ genes in liver-derived cell lines are correlated with single nucleotide changes in the TATA boxes in each promoter (28). Suppression of mouse MT-III gene expression in organs other than the brain has been attributed to CTG triplet repeats within the promoter. However, the binding of a nuclear protein to the CTG sequence has not been demonstrated (29).

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[1] The abbreviations used are: MT, metallothionein; bp, base pair(s); EMSA, electrophoretic mobility shift assay; MRE, metal-responsive element; URE, upstream regulatory element; kbp, kilobase pair.

The participation of specific transcription factors has been proposed as a potential mechanism for the regulation of cell-specific MT transcription. A protein that binds in a metal-dependent manner to CCAAT-homologous sequences in the mouse MT-I promoter has been isolated from rat liver (30). The amino acid sequence of this protein is homologous to members of the liver-enriched C/EBP protein family. However, the function of this protein has not been explored in the context of cell- or tissue-specific regulation of MT transcription. We now report on the identification of UREs and a transcription factor that binds to these elements, which control intestinal cell-specific transcription of *C. elegans* MT genes.

*C. elegans* provides a powerful system for investigating the molecular aspects of cell-specific MT transcription. The developmental and cellular biology of *C. elegans* is thoroughly understood in exceptional detail (31–33). The adult *C. elegans* hermaphrodite contains 959 somatic cells that comprise reproductive, muscular, nervous, and digestive systems (31–33). The timing of cell division and differentiation is invariant, and the developmental lineage of each cell can be traced back to the fertilized oocyte (31). High levels of evolutionary conservation between *C. elegans* and other organisms are observed in many signal transduction, gene regulatory, and developmental pathways (34–37). In addition, stable lines of transgenic nematodes that express reporter transgenes in cell-specific and temporal patterns that mimic endogenous genes can easily be generated (38, 39).

Two MT genes, designated *mtl-1* and *mtl-2*, have been identified and characterized in *C. elegans* (22). Induction of *mtl-1* and *mtl-2* transcription, following metal exposure, occurs exclusively in all intestinal cells at post-embryonic stages of development. Furthermore, significant levels of intestinal cell transcription are not observed in the absence of stress (22). These observations suggest that both tissue-specific and metalloregulatory factors control *C. elegans* MT expression.

Several non-metal inducible *C. elegans* genes are also expressed exclusively in intestinal cells. These include the six vitellogenin genes, *vit-1* to *vit-6*, the cysteine protease *cpr-1*, and the gut esterase *ges-1* (40–42). Multiple copies of heptameric elements, which have the consensus sequence CTGATAA, are present in the promoters of each of these genes, as well as the two *C. elegans* MT genes (22, 41, 43, 44). Originally identified in the upstream regulatory regions of the *C. elegans* vitellogenin genes, these elements are believed to be responsible for controlling the transcription of intestinal cell-specific genes (44, 45). Deletion or mutation of the heptameric elements, in reporter transgenes containing *vit-2, ges-1,* or *cpr-1* promoters, causes either a loss of intestinal cell reporter gene expression or expression in non-intestinal cells (41, 43, 44, 46).

The nucleotide sequence of the heptameric element is identical to the consensus-binding site for the GATA family of transcription factors (GATA factors). GATA factors constitute a family of structurally related transcription factors that interact with the (A/T)GATA(A/G) consensus sequence. GATA factors are expressed in distinct developmental and tissue-specific patterns, and their involvement in the regulation of cell-specific gene transcription is well established (reviewed in Simon (47) and Evans (48)).

Several GATA factors have been isolated from *C. elegans*: ELT-1, ELT-2, ELT-3, and END-1 (49–52). The expression of ELT-2 is restricted to intestinal cells in embryonic and post-embryonic stages of *C. elegans* development. Immunofluorescence, mutagenesis, and ectopic expression experiments suggest that it is a regulator of intestinal cell-specific gene transcription. Furthermore, the intestine is not properly formed in *C. elegans* in which the *elt-2* gene has been disrupted (45). The cell-specific pattern of post-embryonic ELT-2 expression is identical to those of both *C. elegans* MT genes. Thus, ELT-2 is a potential regulator of *mtl-1* and *mtl-2* transcription.

Analysis of the 5′-regulatory regions of the *C. elegans* MT genes reveals the presence of two GATA-like elements in the *mtl-1* promoter and five in *mtl-2* (Table I). In this report, the contribution of each of these elements in the regulation of *C. elegans* MT transcription is examined. In addition, the ability of the ELT-2 transcription factor to bind to the GATA elements in the *C. elegans* MT promoters (*a*) and effect MT transcription *in vivo* (*b*) is investigated. GATA elements and ELT-2 are required for *mtl-1* and *mtl-2* transcription. In addition, ectopic expression experiments indicate that ETL-2 alone, in the absence of added metal or stress, is sufficient to induce *C. elegans* MT transcription. Here we report on the roles of GATA elements and the associated transcription factor in controlling cell-specific MT transcription. Data suggest that other factors (*i.e.* metal sensor) contribute to the metal-inducible expression of the *C. elegans* MT gene. It has not previously been reported that GATA elements or transcription factors regulate MT transcription. Thus, the results presented in this report provide novel insights into the complex mechanisms that govern the expression of MT genes.

## EXPERIMENTAL PROCEDURES

*Growth and Culture of C. elegans*—*C. elegans* were routinely maintained at 20 °C on NGM agar (1.7% agar, 25 mM potassium phosphate; pH 6.0, 50 mM NaCl, 2.5 μg/ml peptone, 5 μg/ml cholesterol, 1 mM $MgCl_2$, 1 mM $CaCl_2$) plates seeded with *Escherichia coli* strain OP50 as a food source (53). *C. elegans* used for inoculation of liquid cultures were grown on 100-mm NGM plates and then removed by washing with M9 buffer (22 mM $KH_2PO_4$, 42 mM $Na_2HPO_4$, 85 mM NaCl, 1 mM $MgSO_4$) (54). Nematodes were then added to complete S medium (0.1 M NaCl, 50 mM potassium phosphate, pH 6.0, 5 μg/ml cholesterol, 10 mM potassium citrate, 3 mM $CaCl_2$, 3 mM $MgSO_4$, 50 μM EDTA, 25 μM $MnCl_2$, 10 μM $FeSO_4$, 10 μM $MnCl_2$, 10 μM $ZnSO_4$, 1 μM $CuSO_4$), which contained *E. coli,* and grown at 20 °C with constant agitation (53).

*Deletion Analysis*—Deletion analysis was used to determine the minimal DNA sequence 5′ of the *mtl-1* and *mtl-2* transcription start sites necessary to confer cadmium-inducible, cell-specific, and developmentally regulated transcription *in vivo*. This analysis was accomplished by creating a series of reporter transgenes in which successively larger regions of the *mtl-1* and *mtl-2* promoters were deleted. The reporter transgenes consisted of MT promoter fragments that were inserted into *C. elegans* expression vectors. These vectors express β-galactosidase (*lacZ*) that is fused to the nuclear targeting sequence of the SV40 large T antigen. The *mtl* reporter transgenes were subsequently used to generate transgenic *C. elegans* (22, 38, 55). Levels and cell-specific patterns of cadmium-inducible β-galactosidase expression were compared between transgenic nematodes that contain reporter transgenes regulated by promoter fragments of varying lengths.

To prepare *mtl-1/lacZ* reporter transgenes, promoter fragments were excised from the *mtl-1/lacZ* fusion construct pMT1.1 (22). pMT1.1 contains an ~1.7-kbp of *mtl-1* that is immediately upstream of the initiator ATG. Digestion of pMT1.1 with *Sac*II and *Pml*I released an ~1.3-kbp DNA fragment from the 5′-end of the upstream regulatory region. The 3′-overhang generated following the *Sac*II digestion was removed following incubation with T4 DNA polymerase (56). After the blunt ends were joined, a reporter transgene regulated by 366 bp of *mtl-1* promoter DNA was produced. This construct was designated p366*mtl-1/lacZ*. A second construct was prepared by incubating pMT1.1 with *Sac*II and *Bsa*AI, removing the 3′-overhang, and then ligating the blunt ends. This generated a 5′-deletion that removed an additional 46 bp to yield p320*mtl-1/lacZ*. A third transgene was prepared that contained 253 bp of the *mtl-1* promoter, p253*mtl-1/lacZ*. This construct was produced by first incubating pMT1.1 with *Xca*I and then isolating a 3289-bp DNA fragment, which contained 253 bp of the *mtl-1* promoter attached to an ~3-kbp fragment of the *lacZ* reporter gene. This fragment was then joined to a 4188-bp DNA fragment that included the pUC19 backbone vector, the 3′ *unc-54* region, and the remainder of the *lacZ* gene (55). The 4188-bp fragment was isolated following the sequential digestion of pMT1.1 with *Sac*II and treating with T4 DNA polymerase to generate blunt ends and then digesting with *Eco*RV and *Xca*I.

A reporter transgene consisting of a *mtl-2* promoter deletion was

prepared by incubating the *mtl-2/lacZ* fusion construct pMT2.1 with *Pst*I. pMT2.1 contains ~2.3 kbp of the DNA adjacent to the 5′-end of *mtl-2* attached to a *lacZ* reporter gene (22). The *Pst*I digestion released an ~2-kbp fragment from the 5′-end of the upstream regulatory region. Following ligation of the remaining plasmid, the resulting expression vector contained 324 bp of the *mtl-2* promoter region upstream from *lacZ*. This reporter transgene was designated p324*mtl-2/lacZ*. Next, a series of promoter deletions were prepared by removing short regions of DNA from the 5′-end of the upstream regulatory region in p324*mtl-2/lacZ* by exonuclease III digestion (56). In these reporter transgenes, designated p292*mtl-2/lacZ*, p269*mtl-2/lacZ*, p191*mtl-2/lacZ,* p183*mtl-2/lacZ*, p174*mtl-2/lacZ*, and p160*mtl-2/lacZ*, β-galactosidase expression is regulated by 292, 269, 191, 183, 174, and 160 bp of the *mtl-2* promoter, respectively.

*C. elegans* were transformed with the reporter transgenes that contained promoter deletions by microinjecting young adult N2 nematodes with recombinant plasmid DNA and the plasmid pRF4, which encodes the dominant selectable marker *rol-6*(*su1006*), as described previously (22, 38, 57). Transgenic *C. elegans* were selected and the reporter transgenes maintained as extrachromosomal arrays, as described previously (22).

*Mutational Analysis of GATA Elements*—To assess the contribution of each GATA element in the regulation of *mtl-1* and *mtl-2* transcription *in vivo*, individual GATA elements were modified. Promoter fragments that contained modified GATA elements were produced by oligonucleotide-directed mutagenesis (56, 58). To produce uracil-containing, single-stranded DNA templates, genomic DNA fragments of *mtl-1* and *mtl-2* were first cloned into pGEM plasmids (Promega). A 1566-bp *mtl-1* fragment was isolated from pMT1.1 following a *Bam*HI digestion and inserted into pGEM3zf(+), which was cut with the same restriction enzyme. This construct, designated pG3mtl1, contains 1529 bp of the region in *mtl-1* that is upstream from the transcription start site. An ~1.2-kbp *mtl-2* DNA fragment was isolated following the digestion of a genomic *mtl-2* clone (22) with *Pst*I and *Bst*XI and then cloned into pGEM5zf(+), which was cut with the same enzymes. This construct, designated pΔmtl2, includes 324 bp of the 5′-regulatory region. The remainder of the insert consists of the coding region of *mtl-2* (22). *E. coli* strain CJ236 (*dut*$^-$ *ung*$^-$) was transformed with the pGEM plasmids and uracil-containing, single-stranded DNA templates produced by infecting the bacteria with M13K07 helper phage (56).

To generate site-directed mutations in the individual GATA elements, antisense oligonucleotides containing 6–10-bp mismatches, which also encoded unique endonuclease restriction sites, were used to prime complementary strand DNA synthesis. These oligonucleotides contained 8–11 correct nucleotides at the 5′-end of the mismatch nucleotides and 13–18 correct nucleotides at the 3′-end of the mutant sequence (Tables II and III). The sequences of the GATA elements were changed to those previously reported to inhibit GATA-mediated transcription of *vit-2* (44). Following DNA synthesis, *E. coli* DH5α (*dut*$^+$ *ung*$^+$) were transfected and the double-stranded pGEM plasmids that contained the modified promoters were recovered (56, 58). Mutations were confirmed by restriction endonuclease digestion and nucleotide sequencing.

Mutated *mtl-1* promoter fragments were excised from pG3mtl1 vectors following incubation with *Pml*I and *Hin*dIII. A 432-bp *mtl-1* DNA fragment, which includes 366 bp of the *mtl-1* promoter, was inserted into the *C. elegans* expression vector pPD16.51 (55). To generate *mtl-2*-containing *lacZ* expression vectors, pΔmtl2 was first cut with *Bst*XI and *Nco*I and an ~0.9-kbp fragment isolated. This fragment was sequentially cut with *Hpa*II, treated with Klenow DNA polymerase, which filled-in the 5′-overhang, and then cut with *Pst*I. The resulting 450-bp *Pst*I/blunt *mtl-2* fragment contains 324 bp of the *mtl-2* promoter and 150 bp of the structural region of the gene. The structural region includes the 5′-untranslated region, the first exon, the intron, and 33 bp of the second exon. This DNA fragment was inserted into pPD16.51 that was cut with *Pst*I and *Sma*I. This construct expressed a fusion protein consisting of the N-terminal 16 amino acids of MTL-2 and a 7-amino acid linker fused to nuclear targeted β-galactosidase. *mtl-1* and *mtl-2* control expression vectors, p1Control and p2Control, respectively, were prepared by excising non-mutated promoter fragments from the corresponding pGEM vectors using the cloning schemes that are outlined above. *C. elegans* were transformed by microinjecting young adult N2 nematodes with recombinant plasmid DNA and the *rol-6* selectable marker, as described above.

*Ectopic Expression of C. elegans GATA-binding Transcription Factors*—To examine the effects of ELT-2 expression on MT gene transcription, a line of transgenic *C. elegans* was generated that contained two independent transgenes, *hsp-16/elt-2* and *mtl-2/lacZ*. In addition, a second line of transgenic nematodes was developed that contained both the *hsp-16/elt-1* and *mtl-2/lacZ* transgenes. In these lines of transgenic *C. elegans,* heat-shock will induce the ectopic expression of the GATA factors in most of the somatic cells (45, 59). If either ELT-1 or ELT-2 can activate MT transcription *in vivo*, then heat-shock will induce β-galactosidase expression in non-intestinal cells and embryos. Similar methods were used to study the ability of these GATA factors to control *ges-1* transcription in *Drosophila* and mouse (45, 60, 61).

Transgenic *C. elegans* containing the *mtl-2/lacZ* transgene were prepared by first injecting *unc-119*(*ed4*) mutant nematodes with p324*mtl-2/lacZ* and the selectable marker pDPMM016D. The plasmid pDPMM016D encodes the wild type form of *unc-119*. *C. elegans* that contain this transgene will not exhibit the *unc-119* phenotype (38, 62). The transgene was integrated into the *C. elegans* genome following γ-irradiation and integrated lines were outcrossed several times, as described previously (43). The strain of *C. elegans* containing the integrated *mtl-2/lacZ* transgene was designated JF4(*mtl-2/lacZ*). Lines of transgenic *C. elegans* containing either the *hsp-16/elt-1* or *hsp-16/elt-2* expression vector integrated into the genome, designated JM53(*hsp-16/elt-1*) or JM57(*hsp-16/elt-2*), respectively, were prepared as described previously (45).

*C. elegans* containing pairs of transgenes were created by standard genetic crosses. Strains JM53(*hsp-16/elt-1*) and JF4(*mtl-2/lacZ*) were crossed to produce a line of transgenic *C. elegans* that contains both *hsp-16/elt-1* and *mtl-2/lacZ*. This line was designated JF6(*hsp-16/elt-1, mtl-2/lacZ*). Line JF5(*hsp-16/elt-2, mtl-2/lacZ*), which contains both the *hsp-16/elt-2* and *mtl-2/lacZ* transgenes, was generated by crossing JF4(*mtl-2/lacZ*) with JM57(*hsp-16/elt-2*).

*Histochemical Assay for Reporter Gene Transcription*—Transgenic *C. elegans* were grown on a 100-mm NGM plate seeded with *E. coli* for ~4 days at 20 °C. Nematodes were harvested, suspended in 10 ml of S basal medium (54), and then incubated for 8–14 h at 20 °C with constant agitation. For cadmium treatment, $CdCl_2$ was added to a final concentration of 100 μM. Following the incubation, *C. elegans* were collected by centrifugation for 5 min at 1,000 × *g*, washed twice with M9 buffer, frozen in liquid nitrogen, and then lyophilized.

To induce ELT-1 and ELT-2 expression prior to assaying for β-galactosidase activity, transgenic *C. elegans* were heat-shocked by incubating mixed stage nematodes for 1–3 h at 34 °C. The nematodes were allowed to recover by incubating overnight at 20 °C and then collected and lyophilized as described above. Embryos at the 2–4-cell stage were collected from JF5(*hsp-16/elt-2, mtl-2/lacZ*) nematodes and allowed to develop for 6 h at 20 °C. They were then heat-shocked at 34 °C for 30-min and allowed to recover overnight at 20 °C prior to staining.

Lyophilized nematodes and embryos were fixed with acetone and stained for β-galactosidase activity using 5-bromo-4-chloro-3-indolyl-β-galactoside as a chromogenic substrate as described previously (22, 63). *C. elegans* were incubated in staining solutions for 1–2 h, following cadmium treatment, or ~12 h following heat-shock.

*Preparation of C. elegans Extracts*—Nematode extracts were prepared using a modification of the procedure of Land *et al.* (64). Mixed stage populations of N2 *C. elegans* were grown in liquid culture. Cadmium-treated nematodes were grown in 100 μM $CdCl_2$ for 12 h prior to harvest. *C. elegans* were harvested, removed by sucrose flotation, and washed as described previously (22). Washed nematodes were suspended in an equal volume of distilled, deionized water and immediately frozen in liquid nitrogen. Approximately 5 g of frozen *C. elegans* was pulverized in a liquid nitrogen-cooled mortar. Powdered nematodes were suspended in 5 volumes of 20 mM HEPES buffer, pH 7.6, containing 0.5 mM dithiothreitol, 4 μg/ml leupeptin, 4 μg/ml aprotinin, 4 μg/ml pepstatin, 10 mM benzamidine, and 28 μM E-64 immediately before sonication. The suspension was sonicated on ice for 4 min with an ultrasonic disintegrator set at 80% duty cycle using 1-s pulses. Homogenates were centrifuged at 100,000 × *g* for 1 h at 4 °C. The supernatant fraction was collected and then filtered through Miracloth (Calbiochem). Aliquots were immediately frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were determined using the Coomassie Protein Assay Reagent (Pierce).

*Electrophoretic Mobility Shift Assay*—EMSAs were performed to examine the interaction between GATA elements in the *C. elegans* MT promoters and *C. elegans* extract proteins (*a*) and the GATA-binding transcription factor ELT-2 (*b*). Double-stranded oligonucleotide probes were generated by first annealing complementary pairs of single-stranded oligonucleotides. The sequences of the oligonucleotides are identical to regions that included the GATA elements 1.1, 2.1, and 2.4 (Tables I—III). Two to four additional guanidine residues were added to the 5′-end of one of the oligonucleotide strands. The double-stranded oligonucleotides were end-labeled by filling-in the 5′-overhangs with

[$\alpha$-$^{32}$P]dCMP. For filling-in reactions, 10–20 pmol of annealed oligonucleotide were combined with 33 $\mu$M each of dATP, dGTP, dTTP; 50 $\mu$Ci of [$\alpha$-$^{32}$P]dCTP; and 20 units of Klenow fragment in reaction buffer (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 7.5 mM dithiothreitol) and then incubated for 15 min at room temperature. Reactions were terminated by the addition of 10 mM EDTA (final concentration) and incubation at 75 °C for 10 min. Unincorporated nucleotides were separated from the labeled products by Sephadex G-25 spin column chromatography.

DNA-protein binding reactions using *C. elegans* total protein extracts were performed by incubating 5–10 $\mu$g of extract protein with 25–50 fmol of labeled oligonucleotide ($3 \times 10^4$ cpm), 1–5 $\mu$g of poly(dI-dC), and unlabeled competitor oligonucleotide in extract assay buffer (10 mM HEPES, pH 7.6, containing 10% glycerol, 105 mM NaCl, 2 mM $MgCl_2$). Protein and competitor oligonucleotides were incubated for 15 min on ice, prior to the addition of labeled probe. After probe addition, reactions were incubated for 30 min at 25 °C and then placed in ice prior to electrophoresis.

DNA-protein binding reactions were also performed using *in vitro* transcribed and translated ELT-2 protein. ELT-2 protein was synthesized from the full-length *elt-2* cDNA, contained in pBluescript SK$^-$ (50), using the TNT-coupled transcription-translation system (Promega). Binding reactions were performed as described previously (50). Briefly, 1 $\mu$l of the *in vitro* transcription/translation reaction mixture was combined with ~50 fmol of labeled oligonucleotide ($2.5–5 \times 10^4$ cpm), 0.38–0.5 $\mu$g of poly(dI-dC), and unlabeled competitor oligonucleotide in modified Zhang Buffer (25 mM HEPES, pH 7.6, containing 10% glycerol, 50 mM KCl, 10 $\mu$M $ZnCl_2$) (65). Incubations were performed as described above.

Products were resolved by polyacrylamide gel electrophoresis using 6% polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) made in 0.5× TBE (1× TBE contains 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA) that had been pre-electrophoresed for 1 h at 150 mV. Electrophoresis of the reaction mixtures was performed at 30 mA at 4 °C for 1–4 h. Gels were then dried, and the amount of DNA-protein complex that formed was determined by PhosphorImager analysis, using ImageQuant Software version 3.3 (Molecular Dynamics).

## RESULTS

*Deletion Analysis of the mtl-1 and mtl-2 Promoters*—Deletion analysis was used as the initial step in identifying *cis* regulatory elements that control the intestinal cell-specific transcription of the *C. elegans* MT genes. In addition, it served to delimit the 5′-boundaries of the minimal promoters for *mtl-1* and *mtl-2*. Minimal promoters are defined as the shortest region of DNA, upstream from the transcription start site, that can regulate cadmium-inducible, intestinal cell-specific, and developmentally modulated gene transcription.

Larval and adult *C. elegans* containing the p366*mtl-1/lacZ* transgene constitutively expressed $\beta$-galactosidase in three pharyngeal cells. This pattern of constitutive expression is similar to that previously observed (22). Larval *C. elegans* exhibited high levels of cadmium-inducible $\beta$-galactosidase expression, whereas expression was attenuated in adult nematodes. The intestinal cell-specific pattern and levels of inducible reporter gene expression were identical to those previously observed in *C. elegans* that contain the pMT1.1 reporter transgene (Table IV) (22).

When p320*mtl-1/lacZ* nematodes were treated with cadmium, $\beta$-galactosidase expression was induced in the intestinal cells of L1 and L2 larvae. However, *mtl-1* promoter activity in L3, L4, and adult intestinal cells was not observed. The results suggest that the region between −366 and −320 bp contains a regulatory element(s) that controls *mtl-1* transcription in later developmental stages. Transgenic nematodes containing p253*mtl-1/lacZ* constitutively expressed *lacZ* in three pharyngeal cells; however, cadmium exposure did not induce intestinal cell transcription of the reporter transgene. The region between −320 and −253 includes one GATA element, GATA1.1 (Table I), and this result suggests that this element may be involved in the regulation of *mtl-1* transcription.

Transgenic nematodes containing the *mtl-2* reporter transgene, p324*mtl-2/lacZ*, were exposed to cadmium or heat shock.

TABLE I
*Location of GATA elements in the C. elegans metallothionein genes*

| | Gene | Sequence | Position[a] |
|---|---|---|---|
| GATA1.1 | *mtl-1* | CTGATAA | −290 to −284 |
| GATA1.2 | *mtl-1* | TTATCAG[b] | −71 to −65 |
| GATA2.1 | *mtl-2* | CTGATAA | −278 to −272 |
| GATA2.2 | *mtl-2* | TTCTCAG[b] | −230 to −224 |
| GATA2.3 | *mtl-2* | TTATCAG[b] | −101 to −95 |
| GATA2.4 | *mtl-2* | TTATCAG[b] | −65 to −59 |
| GATA2.5 | *mtl-2* | GTGATAG | −81 to −76 |

[a] Positions are relative to the transcription start site in each gene.
[b] Inverse complement of the consensus GATA sequence.

The pattern of inducible *mtl-2* promoter activity was identical to that previously observed in transgenic nematodes carrying the pMT2.1 transgene (22). $\beta$-Galactosidase activity was detected exclusively in the larval and adult intestinal cell nuclei.

In p292*mtl-2/lacZ*, an additional 32 bp of DNA was removed from the 5′-end of the upstream regulatory region, which contains sequences that are homologous to cAMP response and AP-1 UREs (22). Treatment of transgenic *C. elegans* carrying p292*mtl-2/lacZ* with cadmium induced reporter gene expression in intestinal cells of larvae and adults. However, the level of promoter activity appeared to be reduced by ~50% compared with that of transgenic nematodes containing either pMT2.1 or p324*mtl-2/lacZ*. This result suggests that these potential regulatory elements may participate in the transcriptional control of *mtl-2*.

Cadmium-treated and non-treated transgenic *C. elegans* containing p269*mtl-2/lacZ* did not express $\beta$-galactosidase in any cells. This transgene is missing an additional 23 bp from the 5′-end of the upstream regulatory region of *mtl-2*, which includes the GATA2.1 element (Table I). This result suggests that GATA2.1 may be essential for *mtl-2* transcription.

Additional 5′-deletions of the *mtl-2* promoter were also examined (Table IV). $\beta$-Galactosidase activity was not detected in any cells of transgenic nematodes, in either the presence or absence of cadmium.

Deletion analysis demonstrated that all information necessary to control cadmium-inducible, intestinal cell-specific, and developmentally regulated *mtl-1* and *mtl-2* transcription is present within 366 and 324 bp upstream of the transcription start sites, respectively. These fragments were defined as the minimal promoters. In addition, the results suggest that GATA elements are involved in the regulation of *C. elegans* MT gene transcription.

*Site-direction Mutation Analysis of mtl-1 and mtl-2 GATA Elements*—The functional contribution of GATA elements in regulating transcription of the *C. elegans* MT genes was determined by site-directed mutation analysis. The effect of mutating each GATA element on *C. elegans* MT promoter activity was assessed *in vivo* using transgenic nematodes.

*C. elegans* containing a reporter gene in which the GATA1.1 element (−290 to −284) was modified did not express significant amounts of $\beta$-galactosidase following cadmium exposure, relative to levels observed in transgenic nematodes containing the p1 control transgene (Fig. 1). In contrast, mutation of the GATA1.2 element (−71 to −65) had no detectable effect on either the level or pattern of $\beta$-galactosidase expression in cadmium-treated transgenic *C. elegans*. These results suggest that the GATA1.1 element is essential for *mtl-1* transcription.

Cadmium treatment of *C. elegans* carrying transgenes consisting of *mtl-2* minimal promoters in which the sequence of either the GATA2.1 (−278 to −272) or GATA2.4 (−65 to −59) element was modified did not activate gene expression (*i.e.* $\beta$-galactosidase was not detected) (Fig. 1). The levels of cadmium-inducible $\beta$-galactosidase expression in *C. elegans* that con-

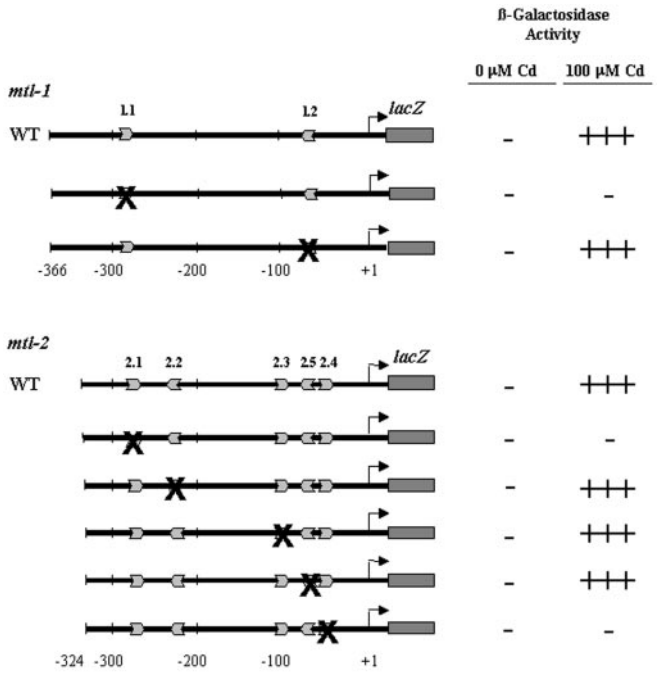



FIG. 1. **Mutational analysis of GATA elements in the *C. elegans* metallothionein promoters.** Potential GATA factor binding-sites in the *mtl-1* and *mtl-2* minimal promoters were modified by site-directed mutagenesis, as described under "Experimental Procedures." The specific GATA sequence that has been modified in each transgene is indicated by ×. Transgenic nematodes were exposed to 100 $\mu$M $CdCl_2$ for 8–12 h (*100 $\mu$M Cd*) and then stained for $\beta$-galactosidase activity. Identical populations of *C. elegans* were not exposed to cadmium (*0 $\mu$M Cd*) prior to staining. $\beta$-Galactosidase activity in *C. elegans* that contain reporter transgenes incorporating mutated promoter sequences is expressed relative to the activity observed in nematodes that contain unmodified reporter transgenes (*WT*). Transcription start sites are indicated by *arrows*. Data represent evaluation of three to five independent experiments for each mutation.

tained reporter transgenes in which the sequence of the remaining three GATA elements, 2.2, 2.3 or 2.5, were changed were identical to that of transgenic p2 control *C. elegans*. This suggests that only GATA2.1 and GATA2.4 are involved in the regulation of *mtl-2* transcription. In addition, both of these elements must be present in order for *mtl-2* transcription to occur.

None of the *mtl-1* or *mtl-2* GATA mutations caused either an increase in $\beta$-galactosidase expression in the absence of metal or expression in non-intestinal cells, suggesting that these UREs participate in activation rather than repression of transcription.

*Binding of C. elegans Extract Proteins to GATA Elements in mtl-1 and mtl-2—In vivo* site-directed mutagenesis analysis identified several GATA elements that are required for cadmium-inducible transcription of *mtl-1* and *mtl-2*. EMSA were performed to determine if *C. elegans* proteins specifically bind to these sequences. A sequence-specific DNA-protein complex was formed when a $^{32}$P-labeled, double-stranded oligonucleotide probe, which included the GATA1.1 sequence (Tables II and III), was incubated with *C. elegans* protein extracts (Fig. 2). Formation of this complex was successfully competed by the addition of a 50-fold molar excess of unlabeled GATA1.1 oligonucleotide (Fig. 2, *lane 3*). The addition of a 50-fold molar excess of an unlabeled oligonucleotide in which the sequence of the GATA1.1 element was changed from CTGATAA to CGGATCC (*i.e.* mutant GATA) did not compete with the binding (Fig. 2, *lane 4*). This modification is identical to that used above in the site-directed mutagenesis analysis in which cadmium-inducible reporter gene expression did not occur. An oligonucleotide, in which a sequence adjacent to the GATA1.1 element was modified, did compete with complex formation (Fig. 2, *lane 5*). In addition, protein-DNA complexes were not produced when nematode extract proteins were incubated with a $^{32}$P-labeled oligonucleotide that contained the mutant GATA1.1 sequence (Fig. 2, *lanes 7–9*).

EMSA were also used to examine protein interactions with the GATA2.1 element from *mtl-2*. A DNA-protein complex was formed between *C. elegans* extract proteins and a labeled oligonucleotide that included the GATA2.1 sequence (Fig. 3, *lane 2*). The formation of this complex was successfully competed by the addition of excess of unlabeled oligonucleotide (Fig. 3, *lanes 3–5*). Addition of up to a 100-fold molar excess of mutant GATA2.1 oligonucleotide (Tables II and III) did not affect complex formation (Fig. 3, *lanes 6–8*). Furthermore, a GATA-specific protein-DNA complex was not generated when *C. elegans* proteins were combined with a labeled oligonucleotide containing the mutant GATA2.1 sequence (Fig. 3, *lanes 10–13*). Similar results were obtained when oligonucleotides that contained the GATA 2.4 sequences were used (data not shown).

These results indicate that *C. elegans* proteins specifically bind to *mtl-1* and *mtl-2* GATA elements. However, no differences in mobility shift patterns were observed in extracts prepared from cadmium-treated *versus* non-treated *C. elegans* (data not shown).

*Binding of ELT-2 to mtl-1 and mtl-2 GATA Elements*—The ability of the *C. elegans* GATA-binding transcription factor ELT-2 to form complexes with GATA1.1, GATA2.1, or GATA2.4 elements was investigated. Oligonucleotide probes that included these GATA sequences were incubated with *in vitro* transcribed/translated ELT-2 protein. ELT-2-DNA complexes were formed in reactions that contained any of the three oligonucleotide probes (Fig. 4). Formation of these complexes was successfully competed by the addition of identical, unlabeled oligonucleotides (Fig. 4, *lanes 4–6*). In contrast, the addition of up to 100-fold molar excesses of unlabeled mutant oligonucleotides, in which GATA sequences were changed from TGATAA to GGATCC, had no effect on complex formation (Fig. 4, *lanes 8–10*). These results demonstrate that ELT-2 binds in a sequence-specific manner to the *mtl-1* and *mtl-2* GATA elements that were shown essential for transcription.

*In Vivo Activation of mtl-2 Transcription by ELT-2*—Several strains of *C. elegans* containing double transgenes were prepared to define further the interaction between the regulation of *C. elegans* MT transcription and GATA factors *in vivo*. When *C. elegans* strain JF5(*hsp-16/elt-2, mtl-2/lacZ*) was heat-shocked, $\beta$-galactosidase expression was detected in hypodermal, muscle, nerve, and pharyngeal cells of L3-L4 larva and adult nematodes (Fig. 5, *A* and *B*). The *mtl-2* gene is not normally transcribed in these cell types (22). This indicates that ELT-2 alone is sufficient to induce *mtl-2* transcription. Interestingly, reporter gene expression was not observed in the intestinal cells of L3-L4 larvae and adult JF5(*hsp-16/elt-2, mtl-2/lacZ*) nematodes. Heat-shock induced reporter gene transcription in the intestinal cells of JF5(*hsp-16/elt-2, mtl-2/lacZ*) embryos (2-fold or later) or L1-L2 larva (Fig. 5, *C* and *D*). In contrast, heat-shock did not induce *mtl-2* transcription in embryos or non-intestinal cells of nematodes that did not ectopically express ELT-2 (*i.e.* JF4(*mtl-2/lacZ*)). Furthermore, the frequency and level of reporter gene expression in the intestinal cells of heat-shocked JF4(*mtl-2/lacZ*) *C. elegans* was 10-fold lower compared with JF5(*hsp-16/elt-2, mtl-2/lacZ*). Exposure of the JF4(*mtl-2/lacZ*) or JF5(*hsp-16/elt-2, mtl-2/lacZ*) strains to cadmium induced reporter gene transcription exclusively in the intestinal cells of post-embryonic nematodes (Fig. 6, *A* and *B*). Activation of *mtl-2* transcription did not occur

TABLE II

*Oligonucleotides used to characterize C. elegans metallothionein GATA elements*

Mutagenic oligonucleotides used as primers in preparing site-direction mutations of GATA sequences in *mtl-1* and *mtl-2* promoter fragments are shown.

| Site | Gene | Sequence[a] |
|---|---|---|
| GATA 1.1 | *mtl-1* | −300 TTGTCAATAAC**G**GAT**CC**AATCAGAAACTAG −279 |
| GATA 1.2 | *mtl-1* | − 81 CCACCCTCTT**GG**ATC**CT**ATATTTGAAGTGGT −51 |
| GATA 2.1 | *mtl-2* | −288 ACACACCTAAC**G**GAT**CC**AGGCCTCTATCAC −259 |
| GATA 2.2 | *mtl-2* | −238 AATTCAAC**GG**CTCCGATATAGCTCAAATC −239 |
| GATA 2.3 | *mtl-2* | −109 CAAAGCTT**GG**ATCCGTGACTGTACATTGTG −78 |
| GATA 2.4 | *mtl-2* | − 75 GCTTGTGCTG**GG**ATCCGCCTGCCTCAAATAG −46 |
| GATA 2.5 | *mtl-2* | − 91 CTGTACATTG**GCGGCC**GC**GG**GTGCTGTTATCAGCTGCC −54 |

[a] Nucleotides that have been changed from the original gene sequence are indicated in bold. The locations of the homologous sequences in the genes are shown relative to the transcription start site.

TABLE III

*Double-stranded oligonucleotides used in gel mobility shift assays*

| Site | Gene | Sequence[a] |
|---|---|---|
| GATA 1.1 WT[b] | *mtl-1* | −299 TGTCAATAACTGATAAAATCAGAAA −275<br>ACAGTTATTGACTATTTTAGTCTTTgggg[c] |
| GATA 1.1 mut | *mtl-1* | −299 TGTCAATAAC**G**GAT**CC**AATCAGAAA −275<br>ACAGTTATTG**C**CTA**GG**TTAGTCTTTgggg |
| GATA 1.1 WT | *mtl-1* | −299 TGTCAATAACTGATAAAATCAGAAACTAGAGCTGTGAC −262<br>ACAGTTATTGACTATTTTAGTCTTTGATCTCGACACTGgg |
| GATA 1.1 mut-G | *mtl-1* | −299 TGTCAATAAC**G**GAT**CC**AATCAGAAACTAGAGCTGTGAC −262<br>ACAGTTATTG**C**CTA**GG**TTAGTCTTTGATCTCGACACTGgg |
| GATA 1.1 mut-M | *mtl-1* | −299 TGTCAATAACTGATAAAATCAG**GCTAGC**GAGCTGTGAC −262<br>ACAGTTATTGACTATTTTAGTC**CGATCG**CTCGACACTGgg |
| GATA 2.1 WT | *mtl-2* | −287 CACACCTAACTGATAAAGGCCTCTA −263<br>GTGTGGATTGACTATTTCCGGAGATgggg |
| GATA 2.1 mut | *mtl-2* | −287 CACACCTAAC**G**GAT**CC**AGGCCTCTA −263<br>GTGTGGATTG**C**CTA**GG**TCCGGAGATgggg |
| GATA 2.4 WT | *mtl-2* | −75 GCTTGTGCTGTTATCAGCTGCCTCA −51<br>CGAACACGACAATAGTCGACGGAGTgggg |
| GATA 2.4 mut | *mtl-2* | −75 GCTTGTGCTG**G**GAT**CC**GCTGCCTCA −51<br>CGAACACGAC**C**CTA**GG**CGACGGAGTgggg |

[a] The location of the GATA elements are indicated by the underlines.

[b] WT, wild type; Mut, mutant.

[c] Guanidine residues that are presented in lowercase letters are the additional nucleotides that were added for subsequent labeling by filling-in reactions.

in either strain in the absence of metal or heat-shock (Fig. 6, *C* and *D*).

Heat-shock treatment of *C. elegans* strain JF6(*hsp-16/elt-1* and *mtl-2/lacZ*), which ectopically expresses ELT-1, predominantly activated *mtl-2* transcription in intestinal cells. The level of intestinal cell expression was comparable to that of heat-shocked JF4(*mtl-2/lacZ*) nematodes. Ectopic expression of ELT-1 did not activate *mtl-2* transcription in embryonic intestinal cells. Reporter gene expression in non-intestinal cells (*i.e.* body wall and pharynx muscle and hypodermal cells) was observed in 1–2% of the β-galactosidase-expressing JF6(*hsp-16/elt-1, mtl-2/lacZ*) *C. elegans*. However, the levels and frequency of expression were considerably lower than those observed in the JF5(*hsp-16/elt-2, mtl-2/lacZ*) strain and are not considered significant. These results suggest that ELT-1 does not contribute to the regulation of *C. elegans* MT transcription.

An additional strain of *C. elegans*, JF7(*mtl-2/lacZ*, *elt-2*$^{-/+}$), was created through a genetic cross of JF4(*mtl-2/lacZ*) with the homozygous null mutant *elt-2*(*ca15*) (45). *C. elegans* with *elt-2*$^{-/+}$ genotype was identified by polymerase chain reaction of genomic DNA isolated from individual nematodes using primers that flank *elt-2* (45). Single JF7(*mtl-2/lacZ*, *elt-2*$^{-/+}$) nematodes were placed on NGM agar plates, which contained 100 μM $CdCl_2$, and allowed to grow for ~3 days. The progeny were then stained for β-galactosidase activity. Of the 357 nematodes examined, 165 (46%) expressed the reporter gene in the intestinal cells. These *C. elegans* were either *elt-2*$^{-/+}$ or *elt-2*$^{+/+}$, as determined by the proper development of the intestinal lumen (45) (Fig. 7*B*). Fifty-seven nematodes were *elt-2*$^{-/-}$, as determined by the improperly formed intestine. The majority of these nematodes did not express β-galactosidase (Fig. 7*A*). Five of the *elt-2*$^{-/-}$ *C. elegans* showed reporter gene expression in one or two intestinal cells. However, the level of β-galactosidase expression was substantially lower than that observed in *elt-2*$^{-/+}$ or *elt-2*$^{+/+}$ *C. elegans* and is not significant. When the JF4(*mtl-2/lacZ*) strain was grown on cadmium-containing NGM plates, only ~55% of the progeny expressed the transgene in the intestinal cells.

The ability of ectopically expressed ELT-2 to activate *mtl-2* transcription (*a*) and the lack of *mtl-2* transcription in *elt-2*$^{-/-}$ *C. elegans* (*b*) indicate that this GATA factor regulates *C. elegans* MT transcription *in vivo*.

## DISCUSSION

The ability of transition metals, as well as other physiologic stressors, to induce MT expression has been established for decades (1). Two models have been proposed that describe how metals induce MT transcription (25, 26). However, metal exposure alone is not sufficient to activate expression. For example, cadmium accumulates in the prostate of metal-exposed rats, but MT expression is not induced in this tissue (66). Thus, cell-specific processes are also involved in regulating MT gene expression.

A comparison among the nucleotide sequences of *mtl-1* and *mtl-2*, and other *C. elegans* genes whose expression is limited to intestinal cells, reveals the presence of multiple potential binding sites for GATA family transcription factors (Table I). Because GATA elements are involved in the regulation of cell-specific gene expression in a variety of organisms, they may also participate in the intestine-specific expression of *mtl-1* and

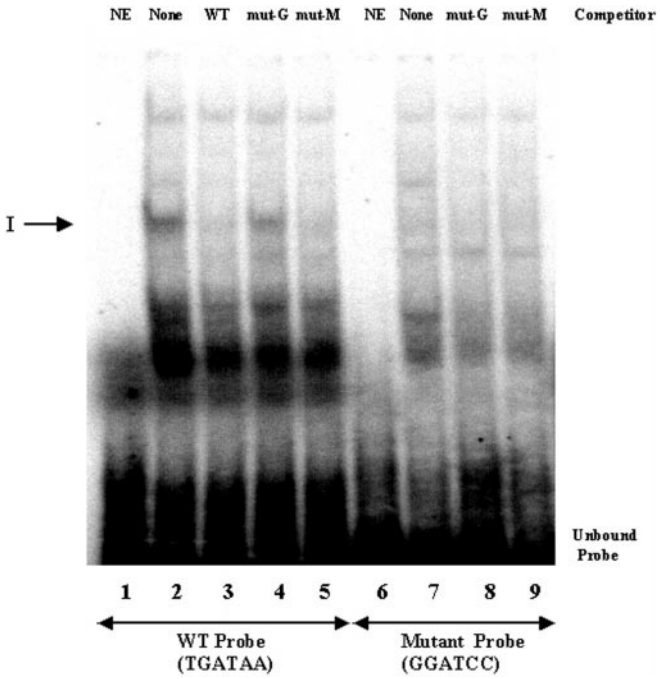



FIG. 2. **Electrophoretic mobility shift analysis of the GATA1.1 sequence and *C. elegans* extract proteins.** A $^{32}$P-labeled, double-stranded oligonucleotide probe (38 bp, Tables II and III), which contained an unmodified (*WT Probe*, *lanes 1–5*) or mutated (*Mutant Probe*, *lanes 6–9*) GATA1.1 sequence, was incubated with 10 $\mu$g of *C. elegans* extract protein. Each probe was incubated in either the absence (*None, lanes 2* and *7*) or the presence of a 50-fold molar excess of unlabeled competitor. Wild type oligonucleotide (*WT, lane 3*), an oligonucleotide in which the GATA sequence was changed from CTGATAA to CGGATCC (*mut-G*; *lanes 4* and *8*), or an oligonucleotide containing a mutation adjacent to the GATA sequence (*mut-M*; *lanes 5* and *9*) were used. A single, sequence-specific protein-DNA complex (*I*) is evident. *NE*, no extract (*lanes 1* and *6*).

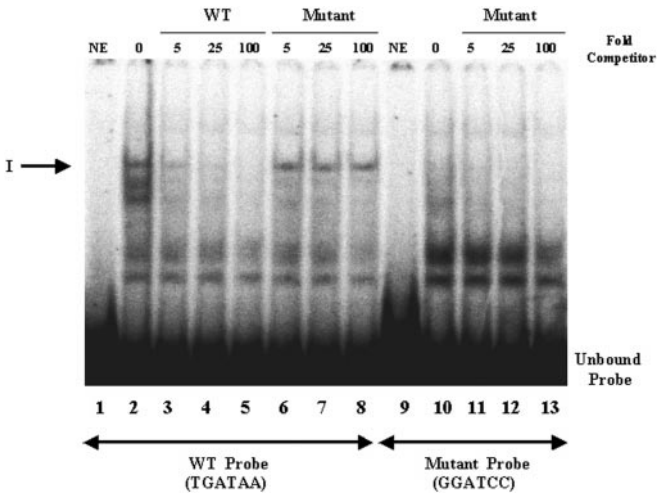



FIG. 3. **Electrophoretic mobility shift analysis of the GATA2.1 element and *C. elegans* extract proteins.** A $^{32}$P-labeled, double-stranded wild type (*WT Probe*, *lanes 1–8*) or mutated (*Mutant Probe*, *lanes 9–13*) GATA2.4 oligonucleotide (Tables II and III) was incubated with 10 $\mu$g of *C. elegans* extract protein. Unlabeled *WT* (*lanes 3–5*) or *Mutant* (*lanes 6–8*, and *10–13*) competitor oligonucleotides were added in 0–100-fold molar excess. A single sequence specific band (*I*) is evident only in the samples containing labeled wild type probe (*lanes 2, 3* and *6–8*). *NE*, no extract (*lanes 1* and *9*).

*mtl-2*. *In vivo* deletion and site-directed mutagenesis analyses confirm that one of these elements in *mtl-1* and two in *mtl-2* are required for cadmium-inducible MT transcription (Table IV and Fig. 1).

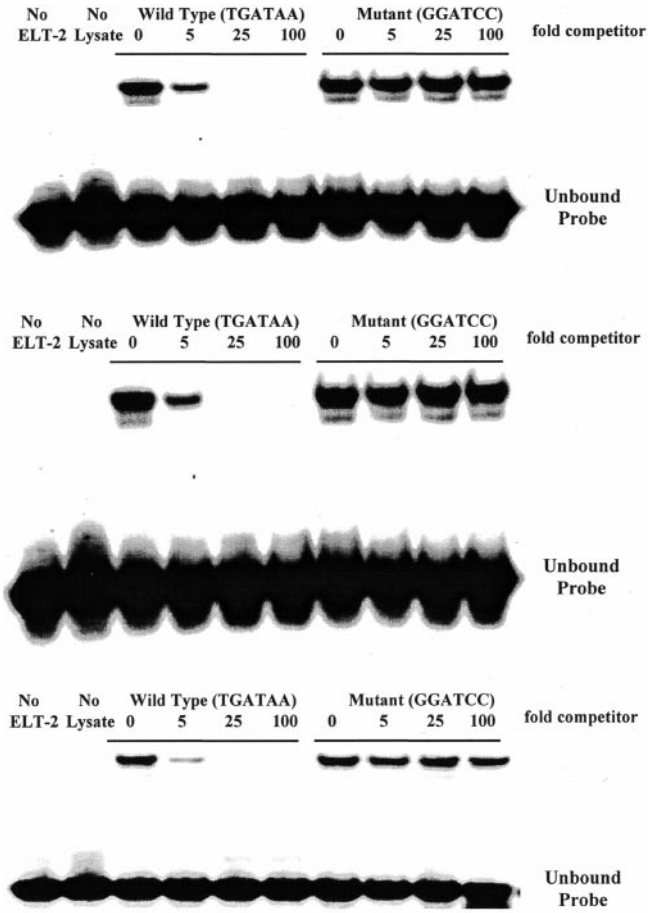



FIG. 4. **Binding of *in vitro* synthesized ELT-2 to *mtl-1* and *mtl-2* GATA elements.** $^{32}$P-Labeled, double-stranded, 25-bp oligonucleotide probes (Tables II and III) that included the GATA 1.1 sequence (*upper panel*), GATA2.1 sequence (*middle panel*), or GATA2.4 sequence (*lower panel*) were incubated with *in vitro* transcribed/translated ELT-2 protein. A molar excess of unlabeled, double-stranded wild type or mutant competitor oligonucleotides were included in the reactions at the levels indicated.

Six families of vertebrate GATA-binding transcription factors, GATA-1 through GATA-6, have been identified. Each has distinct but overlapping tissue-specific patterns of expression. Members of the vertebrate GATA-4/5/6 family are expressed in intestinal cells. The factors may be involved in gastrointestinal development (*a*) and may regulate intestinal cell-specific gene expression (67–74) (*b*). The DNA binding domain of ELT-2 is most closely related to the vertebrate GATA-5 factor (50).

Four GATA factors have been isolated from *C. elegans*: ELT-1, ELT-2, ELT-3, and END-1. The expression of ELT-1 and ELT-3 is limited to embryonic epidermal and hypodermal cells, respectively (51, 52). The GATA factor END-1 is involved in the development of the nematode intestine. *C. elegans* intestinal cells arise from a single progenitor cell, E cell, which is formed at the 8-cell stage of embryogenesis (75). END-1 is only detected between the 1E and 4E cell stages of development and functions during early intestine formation (49). The pattern of *end-1* expression does not overlap those of *C. elegans* MT genes. Thus, ELT-1, ELT-3, and END-1 are not likely to be regulators of *C. elegans* MT transcription.

In contrast, Elt-2 protein and mRNA are first detected at the 2E-cell stage and are present in all of the intestinal cells. They are continuously expressed throughout development and in the adult nematode (50). The post-embryonic pattern of *elt-2* expression is identical to those of *mtl-1* and *mtl-2* (*i.e.* exclusively

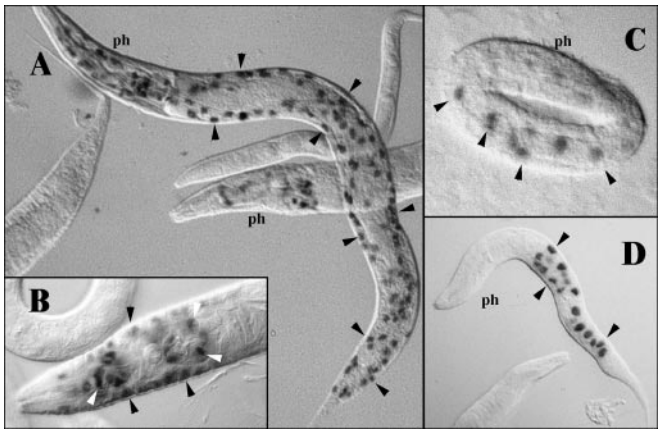



FIG. 5. **Transcription of *mtl-2* in *C. elegans* ectopically expressing ELT-2.** *C. elegans* strain JF5(*hsp-16/elt-2, mtl-2/lacZ*) was heat-shocked at 34 °C and then stained for β-galactosidase activity (see "Experimental Procedures"). *A* shows adult hermaphrodite nematodes with stained body wall and pharyngeal muscle and hypodermal cell nuclei. *Arrowheads* indicate the location of stained hypodermal and body wall muscle nuclei. *B* is an enlargement of a stained adult pharynx. *Black arrowheads* indicate the location of stained hypodermal nuclei. *White arrowheads* identify stained pharyngeal muscle cell nuclei. *C* and *D,* reveal *mtl-2* promoter activity in the intestinal cells of a late embryo and L1 larva, respectively. *Arrowheads* in these *two panels* indicate intestinal cell nuclei that are expressing the reporter transgene. *ph* signifies the location of the *C. elegans* pharynx.

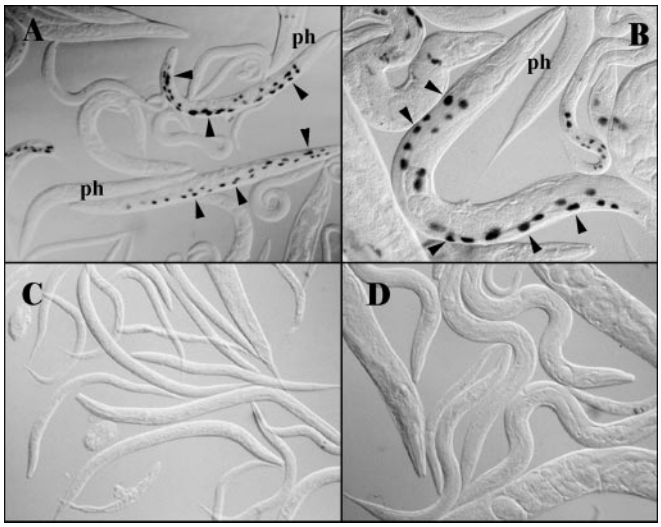



FIG. 6. **Cell-specific expression of the *mtl-2/lacZ* transgene.** The patterns of β-galactosidase expression in mixed populations of *C. elegans* strains JF4(*mtl-2/lacZ*) (*A* and *C*) and JF5(*hsp-16/elt-2, mtl-2/lacZ*) (*B* and *D*). Nematodes were exposed to 100 μM cadmium for 24 h (*A* and *B*) or non-induced (*C* and *D*) prior to staining. *A* and *B* show reporter gene activity exclusively in intestinal cells of adults and larvae. *Arrowheads* in *A* and *B* identify some of the intestinal cell nuclei that are expressing the reporter transgene. *ph* signifies the location of the *C. elegans* pharynx.

in intestinal cells), which suggests that it may be a regulator of MT gene expression.

Electrophoretic mobility shift assays, using *C. elegans* protein extracts, confirms that nematode protein(s) bind to the *mtl-1* and *mtl-2* GATA elements, which were shown by *in vivo* mutagenesis analysis to be required for MT transcription (Figs. 2 and 3). *In vitro* expressed ELT-2 forms a sequence-specific complex with these GATA elements (Fig. 4). These results confirm that ELT-2 binds to the *C. elegans* MT GATA elements. However, they do not conclusively demonstrate that ELT-2 regulates MT transcription.

Ectopic expression of ELT-2 activates *mtl-2* transcription in

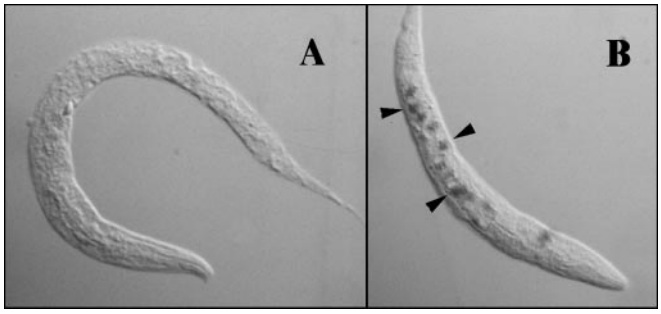



FIG. 7. **Transcription of *mtl-2* in *elt-2(ca15)* null mutant *C. elegans*.** The progeny from a single JF7(*mtl-2/lacZ*$^{+/+}$, *elt-2*$^{-/+}$) nematode were exposed to 100 μM cadmium and then stained for β-galactosidase activity. Nuclear staining of the intestinal cells is not evident in an *elt-2*$^{-/-}$ nematode (*A*). In contrast, in an *elt-2*$^{\pm}$ *C. elegans* (*B*) intestinal cells actively transcribe *mtl-2.* Both nematodes are L1 larva. *Arrowheads* in *A* identify the intestinal cell nuclei that are expressing the reporter transgene.

the absence of metal exposure (Fig. 5, *A–D*). This result indicates that ELT-2 regulates *C. elegans* MT transcription. Similar results were obtained in studies of the gut esterase gene, *ges-1.* Embryonic expression of GES-1 is limited to the E cell lineage (42, 46). When ELT-2 is ectopically expressed, however, GES-1 is observed in most of the cells in the embryo (45).

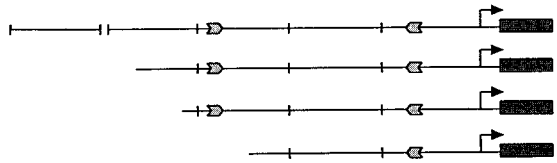
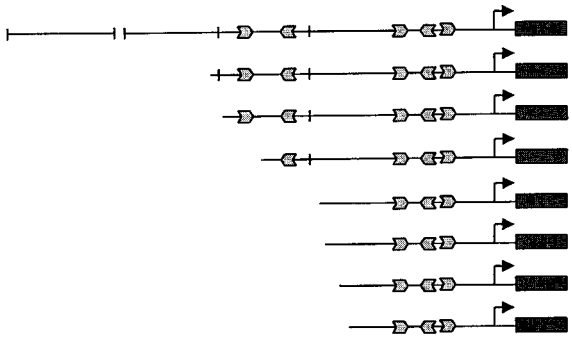
Heat-shock of JF5(*hsp-16/elt-2, mtl-2/lacZ*) nematodes activates *mtl-2* transcription in intestinal cells of embryos at later stages of development (Fig. 5*C*) (*a*); intestinal cells of L1–L2 larvae (Fig. 5*D*) (*b*); and hypodermal, pharynx, muscle, and nerve cells of L3-L4 larvae and adults (Fig. 5, *A* and *B*) (*c*). The pattern, level, and frequency of *mtl-2* transcription in nematodes that express ELT-2 in all cell types are significantly different than those observed in cadmium-treated animals. In addition, heat-shock of the JF4(*mtl-2/lacZ*) strain does not induce significant levels of *mtl-2* transcription. These results indicate that ELT-2 alone is sufficient to activate MT transcription, *i.e.* elevated concentrations of metal are not necessary to induce transcription.

Some characteristics of GATA elements and ELT-2 resemble those of MREs and MTF-1. For example, deletion or modification of specific GATA-sequences in *mtl-1* or *mtl-2* prevents metal-inducible transcription *in vivo* (Fig. 1). Likewise, deletion or modification of specific MREs will prevent metal-inducible transcription of mammalian MTs (76). However, data indicate that GATA elements and ELT-2 do not control metal responsiveness. GATA elements and ELT-2 are responsible for determining the intestinal cell specificity of MT transcription. GATA elements are found in the upstream regulatory regions of *C. elegans* intestinal cell-specific genes including vitellogenins, gut esterase, P-glycoprotein, and cysteine protease (41, 44, 46, 77). Metal-inducible transcription of these genes has not been reported, suggesting that GATA sequences do not function as *C. elegans* MREs. In addition, cadmium exposure does not induce the transcription of a GATA element containing intestinal cell-specific aspartic protease.[2] ELT-2 contains a single zinc finger domain; however, mouse and human MTF-1 contain multiple zinc fingers (25, 50). A characteristic of MTF-1, and other mammalian metal-responsive transcription factors, is the ability of low concentrations of EDTA to inhibit MTF-1-DNA complex formation (25, 78–80). The binding of ELT-2 to GATA elements is significantly less sensitive to EDTA than MTF-1 binding to MREs.[3]

The transcription of *C. elegans* MT genes is limited to intestinal cells as a consequence of the presence of GATA elements

[2] J. H. Freedman, unpublished observations.
[3] J. H. Freedman and L. H. Moilanen, unpublished observations.

TABLE IV
*Deletion analysis of the C. elegans metallothionein promoters*

| Scheme [a] | Reporter Transgene | Promoter Length (bp) | β-Galactosidase Activity [b] | |
|---|---|---|---|---|
| | | | 0 μM Cd | 100 μM Cd |
| *mtl-1* | | | | |
|  | pMT1.1 | ~1700 | - | ++++ |
| | p366*mtl-1/lacZ* | 366 | - | ++++ |
| | p320*mtl-1/lacZ* | 320 | - | ++++ [c] |
| | p253*mtl-1/lacZ* | 253 | - | - |
| *mtl-2* | | | | |
|  | pMT2.1 | ~2300 | - | ++++ |
| | p324*mtl-2/lacZ* | 324 | - | ++++ |
| | p292*mtl-2/lacZ* | 292 | - | ++ |
| | p269*mtl-2/lacZ* | 269 | - | - |
| | p191*mtl-2/lacZ* | 191 | - | - |
| | p183*mtl-2/lacZ* | 183 | - | - |
| | p174*mtl-2/lacZ* | 174 | - | - |
| | p160*mtl-2/lacZ* | 160 | - | - |

[a] The location of potential GATA elements are indicated by the gray arrowheads. Transcription start begins at +1 and the "β-galactosidase reporter gene (lacZ) is indicated by the gray box.
[b] β-Galactosidase activity in transgenic *C. elegans* that contain reporter transgenes containing shortened *mtl-1* and *mtl-2* promoters is relative to the level observed in transgenic nematodes that contain the pMT1.1 and pMT2.1 transgenes, respectively.
[c] The level of β-galactosidase activity in L1 and L2 *C. elegans* is comparable to that observed in transgenic *C. elegans* that contain pMT1.1. No expression however is observed in L3–L4 nematodes.

in the promoters (*a*) and ELT-2 being expressed exclusively in intestinal cells (*b*). Thus, intestinal cell-specific expression is controlled simply by limiting the expression of an essential transcription factor to this specific cell lineage. ELT-2 can activate MT transcription in the absence of metal exposure (Fig. 5), and it is constitutively expressed in intestinal cells in embryonic and post-embryonic stage of development (45, 50). However, *mtl-1* and *mtl-2* are not usually transcribed unless the nematodes are exposed to metal. This indicates that additional regulatory processes contribute to the control of metal-inducible MT gene expression. Since ELT-2 is not the "metal sensor," additional factors must act to regulate the metal inducibility. These factors may directly interact with ELT-2. Vertebrate GATA-factors interact with a variety of proteins, including AP-1, SP-1, and YY-1 (81–87). This interaction can result in either the stimulation or repression of transcription. Alternatively, UREs that bind metal-responsive transcription factors independently of GATA factors may be present in the promoters of the *C. elegans* MT genes.

ELT-2 expression in embryos failed to induce *mtl-2* transcription in intestinal cells before the 2-fold stage of development. Previous studies have shown that heat-shocked JM57(*hsp-16/elt-2*) express ELT-2 protein in most of the cells in early embryos. Ectopically expressed ELT-2 induces the expression of other intestinal cell-specific genes in non-intestinal cell lineages (45). Thus, although ELT-2 alone appears to be sufficient to active *mtl-2* transcription, additional processes must participate in determining the correct developmental pattern of expression. Developmentally modulated MT expression has been described in a variety of other species. Transcription of the *Drosophila Mtn* gene is not detected until the beginning of germ band retraction in endodermal gut primordia (88). In mice, MT-IV is not detected prior to day 7 postpartum (17). Furthermore, MT is not detected in human fetal brain in less than the 35-week-old fetus (89). The mechanisms responsible for developmental regulation of MT transcription have not been elucidated.

Several observations are consistent with a model for the regulation of metal-inducible, intestinal cell-specific *C. elegans* MT gene expression that incorporates a metal-sensitive repressor protein. First, intestinal cell expression of *mtl-2* in heat-shocked L3–L4 and adult JF5(*hsp-16/elt-2, mtl-2/lacZ*) nematodes is infrequent and weak compared with other cell types and the levels observed in cadmium-treated nematodes. Second, although ELT-2 alone activates MT transcription independent of cell type or metal exposure, and it is constitutively expressed in the intestinal cells, the MT genes are only transcribed following metal exposure. In the model, a repressor protein inhibits the ability of ELT-2 to constitutively activate MT transcription. Since cadmium treatment did not affect the binding of ELT-2 to the GATA elements, the metalloregulatory protein would not inhibit ELT-2 binding, rather it prevents ELT-2-mediated transcriptional activation of the *C. elegans* MT genes. In the presence of metal, repression is released and then ELT-2 can activate transcription in the intestine.

Repressor-mediated regulation of stress-inducible gene tran-

scription has been described for several genes. Palmiter (26) has hypothesized that a zinc-sensitive inhibitor blocks MTF-1 binding to MREs to prevent MT transcription. It has been reported that overexpression of the p80 subunit of the protein Ku suppresses metal-inducible MT-I gene transcription in Rat 1 fibroblast cells. The p80 subunit does not directly inhibit MT-I transcription, but its overexpression may activate other factors that inhibit transcription (90). The transcription factor NF$\kappa$B participates in transcriptional control of genes responsive to oxidative stress (91). In the absence of stress, NF$\kappa$B DNA binding is repressed by the inhibitory factor I$\kappa$B, which forms a complex with NF$\kappa$B. Treatment with pro-oxidants results in the dissociation of the I$\kappa$B from NF$\kappa$B and the subsequent activation of target genes. A mammalian heat-shock factor-1 (HSF1)-binding protein has been identified, HSBP1, that directly interacts with the HSF1. HSBP1 inhibits the binding of HSF1 to heat-shock promoter elements to repress heat-shock protein transcription (92). The overexpression of the *C. elegans* HSBP1 homologue, HSB-1, both inhibits *hsp-16* transcription and reduces the survival of heat-stressed nematodes (92).

The ectopic expression of ELT-2 in late embryos and L1–L2 larvae exclusively activates *mtl-2* transcription in intestinal cells. The lack of expression in non-intestinal cells may be due to the presence of developmental stage-specific regulators, which are not present in older larvae and adult *C. elegans*. The intestinal expression may be the result of relatively high levels of ELT-2 produced in the heat-shocked JF5(*hsp-16/elt-2, mtl-2/lacZ*) nematodes that may overcome or "titrate out" a repressor protein. Further investigations will be necessary to confirm the repressor model and resolve any inconsistencies.

Regulation of gene expression by GATA elements and transcription factors is an evolutionarily conserved process. Since ELT-2, a homologue of vertebrate GATA (50), can bind to *mtl-1* and *mtl-2* UREs and control MT transcription, a similar process may function in higher eukaryotes. Sequence analysis of the upstream regulatory regions in MT genes, from a variety of species, identified multiple copies of the GATA consensus sequence. The GATA sequences are interspersed among consensus MRE sequences in invertebrates (sea urchin and fly), amphibians (frog), fishes (stone loach, rainbow trout, northern pike, and carp), and mammals (rat, mouse, Chinese hamster, sheep, and human). The functionality of GATA elements and transcription factors in regulating the expression of these MT genes has not been examined. GATA elements are also present in several invertebrate MT genes that show highly restricted patterns of expression, including the *Drosophila Mto* gene (4, 20) and sea urchin *spMTA* and *spMTB* genes (18, 19, 93). In addition, the expression patterns of the *Drosophila* GATA factors "serpent" and dGATA-c overlap those for *Mtn* and *Mto* (71, 94, 95). Thus, GATA elements and factors may be components of a evolutionarily conserved mechanism that controls cell-specific transcription of MT genes.

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