

The *Drosophila* Homolog of Mammalian Zinc Finger Factor MTF-1 Activates Transcription in Response to Heavy Metals

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Metallothioneins (MTs) are short, cysteine-rich proteins for heavy metal homeostasis and detoxification; they bind a variety of heavy metals and also act as radical scavengers. Transcription of mammalian MT genes is activated by heavy metal load via the metal-responsive transcription factor 1 (MTF-1), an essential zinc finger protein whose elimination in mice leads to embryonic lethality due to liver decay. Here we characterize the *Drosophila* homolog of vertebrate MTF-1 (dMTF-1), a 791-amino-acid protein which is most similar to its mammalian counterpart in the DNA-binding zinc finger region. Like mammalian MTF-1, dMTF-1 binds to conserved metal-responsive promoter elements (MREs) and requires zinc for DNA binding, yet some aspects of heavy metal regulation have also been subject to divergent evolution between *Drosophila* and mammals. dMTF-1, unlike mammalian MTF-1, is resistant to low pH (6 to 6.5). Furthermore, mammalian MT genes are activated best by zinc and cadmium, whereas in *Drosophila* cells, cadmium and copper are more potent inducers than zinc. The latter species difference is most likely due to aspects of heavy metal metabolism other than MTF-1, since in transfected mammalian cells, dMTF-1 responds to zinc like mammalian MTF-1. Heavy metal induction of both *Drosophila* MTs is abolished by double-stranded RNA interference: small amounts of cotransfected double-stranded RNA of *dMTF-1* but not of unrelated control RNA inhibit the response to both the endogenous dMTF-1 and transfected dMTF-1. These data underline an important role for dMTF-1 in MT gene regulation and thus heavy metal homeostasis.

Heavy metals, both essential and nonessential ones, pose a threat to every organism, and their concentration has to be carefully controlled. For example, copper, zinc, and cobalt have to be enriched if scarce but removed if abundant, while nonessential heavy metals, such as cadmium, have to be detoxified. Most species contain small, cysteine-rich proteins, called metallothioneins (MTs), to bind heavy metals for redistribution and/or detoxification (18, 28, 29, 52). In addition to their role in heavy metal homeostasis, MTs have been found to act as potent radical scavengers, thus contributing to antioxidant defense and cellular redox balance. Accordingly, transcription of the genes for MTs is induced not only by heavy metal load but also by a variety of other stress conditions.

Vertebrates contain four types of MTs, all about 60 to 70 amino acids long, of which MT-I and MT-II are stress induced and expressed in all organs, while MT-III and MT-IV are cell type specific and respond only moderately to heavy metal load (41, 55). The stress-induced MT-I and MT-II genes contain in their promoters multiple copies of so-called metal-responsive element (MRE) sequence motifs with the core consensus sequence TGCRNC (50, 54). The transcription factor that binds to these MREs and confers metal inducibility was cloned in our laboratory and termed MTF-1 (for MRE-binding transcription factor 1 or metal-responsive transcription factor 1) (25, 43, 57). MTF-1, a protein with six characteristic zinc fingers of the C₂H₂ type, is essential for heavy metal response and for embryonic development, since homozygous knockout mouse embryos die on embryonic days 13 to 14 due to liver

decay (23). MTF-1, which also contributes to the activation of genes other than MTs, in resting cells localizes to the cytoplasm and enters the nucleus under stress conditions (47, 53). Zinc induction works by direct metal binding to the MTF-1 zinc fingers (7, 11, 15, 25), but the exact signaling pathways for transcriptional activation remain to be established. Even though other metals, such as cadmium and copper, also activate transcription via MTF-1, activation must be indirect since these metals cannot replace zinc in zinc finger binding (6, 25; B. Zhang and W. Schaffner, unpublished). MTF-1 also mediates response to oxidative stress and hypoxia (16, 23, 38, 59). Recently, researchers isolated the MTF-1 gene from another vertebrate, the Japanese puffer fish *Fugu rubripes*. This MTF-1 turned out to be conserved to the ones of mouse and human, most notably in the DNA-binding zinc finger domain (3; see also reference 17). In order to gain more insights into the mechanisms of cellular defense against heavy metal and other stress, we decided to have a closer look at heavy metal homeostasis in *Drosophila melanogaster*, where a rapid genetic dissection is feasible. While transcriptional regulation of heavy metal homeostasis in yeast and the worm *Caenorhabditis elegans* differs from the one in vertebrates, the system in *Drosophila* promises to yield evolutionary insights of great relevance also to mammals.

In *Drosophila*, the two MT genes characterized to date, designated *Mto* (or *MtnB*) and *Mtn* (or *MtnA*), encode proteins of 43 and 40 amino acids, respectively. *Mto*, transcribed from a TATA-less promoter, is primarily active during embryogenesis, while *Mtn*, characteristic for late embryos, larvae, and adult flies, is strongly expressed in the gut, Malpighian tubules, and fat body and also in hemocytes (8, 9, 19, 35, 36, 37, 51). Even though the overall protein sequences of both *Drosophila* MTs deviate considerably from the ones of mammals, their promot-

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ers contain sequence motifs corresponding to mammalian MREs (20, 51). In support of this notion, a promoter fragment from *Mtn* containing two MREs was responsive to zinc after transfection into cultured hamster cells (39). This indicated to us that, if not the MTs themselves, at least the mechanism of their induction should be conserved, and prompted a search for a *Drosophila* homolog of the mammalian MTF-1. A *Drosophila* cDNA encoding a protein reminiscent of vertebrate MTF-1 was found in the expressed sequence tag database. Isolation of cDNAs and of the genomic locus, sequence comparisons, and functional tests reveal that *Drosophila* harbors, some differences in structure and properties notwithstanding, a functional homolog of the vertebrate stress regulator MTF-1. *Drosophila* MTF-1 (dMTF-1) can activate transcription from the promoters of MTs, *Mtn* and *Mto*, and in cultured *Drosophila* cells confers particularly strong transcriptional responses to copper and cadmium.

MATERIALS AND METHODS

Isolation of the phages containing the dMTF-1 gene. A *Drosophila* λ phage genomic library was probed with an EST cDNA (BDGP clone identification, SD03560) containing a putative MTF-1 sequence labeled by random hexamer priming using [α - 32 P]dCTP. Filters were hybridized according to the method described by Church and Gilbert (12). Forty positive clones were obtained, and eight of them were chosen for rescreening and further analysis. Subsequently, all eight positive clones were mapped by standard restriction enzyme digestion and Southern blot analysis (46). The overlapping positive fragments were subcloned into pBluescript SK(-) (Stratagene) and sequenced.

cDNA library screening and isolation of cDNA for dMTF-1. dMTF-1 cDNA was isolated from a cDNA library derived from 8- to 12-h-old *Drosophila* embryos (22), using the same probe as for the screening of the genomic library. To determine the 5' end of dMTF-1 transcripts, rapid amplification of cDNA ends (RACE) was performed with RNA from 0- to 20-h-old *Drosophila* embryos using specific primers for dMTF-1 (SMART RACE kit; Clontech).

Poly(A) RNA isolation and Northern blot. Poly(A) RNA was isolated with oligo(dT) cellulose, and Northern blot analyses were performed according to the method described by Sambrook et al. (46), using approximately 5 μ g of poly(A) RNA per slot.

Generation of OVEC reporter constructs and expression vectors. The reporter and reference plasmids used in the transient transfection assays were generated based on the OVEC-1 reporter system, which is based on quantitative S1 nuclease mapping of transcripts (58). The OVEC reporter constructs containing mouse MT-I (mMT-I) promoter or four copies of a mouse MRE (4 \times mMREd) used in this study are described by Radtke et al. (43). The OVEC reporter genes containing *Drosophila* MT *Mtn* promoter or *Mto* promoter were generated by PCR from the promoter of *Mtn* (-373 to -39 relative to transcription start site) or of *Mto* (-169 to +11 relative to transcription start site) and were inserted into the *SacI/SalI*-digested OVEC vector. The synthetic "minipromoter" construct was generated by insertion of a synthesized oligonucleotide containing the four MREs found in *Mto* promoter in a tandem array (-160 to -143, -133 to -116, -71 to -54, and -12 to +5) into the OVEC vector as done before (57). The human MTF-1 (hMTF-1) expression vector driven by the cytomegalovirus (CMV) promoter (used in mammalian cells) is described by Heuchel et al. (25). The hMTF-1 (with vesicular stomatitis virus tagged at its C-terminal) expression vector used in *Drosophila* Schneider-2 (S2) cells was generated by subcloning hMTF-1 cDNA-vesicular stomatitis virus fragment into the pAc5* vector containing the *Drosophila* *actin 5C* promoter or the pT vector containing the *Drosophila* *tubulin α* promoter. After reconstruction of the full-length dMTF-1 open reading frame in pBluescript SK(-), the entire cDNA was subcloned into pcDNA I (Invitrogen) containing the CMV promoter or pAc5* and pT vectors and was used as the expression vectors for dMTF-1 in mammalian or *Drosophila* cells, respectively.

dsRNA preparation. Double-stranded RNA (dsRNA) was prepared according to Kennerdell and Carthew (31). Briefly, the templates for in vitro transcription of dsRNA were generated by PCR using primers which are flanked by a T7 promoter sequence at the 5' end. These primers specifically amplified a cDNA fragment of 512 bp corresponding to the N-terminal part of dMTF-1 (including the first 104 amino acids) and of 498 bp corresponding to part of the coding

region of *lacZ* (amino acids 6 to 171), respectively. The dsRNAs were synthesized using T7 RNA polymerase (New England BioLabs) followed by DNase I digestion, ethanol treatment, and ethanol precipitation. The quality and amount of the dsRNA were controlled by using agarose gel electrophoresis.

Cell culture. Human embryonic kidney 293 cells, as well as dko7 cell line derived from mouse embryonic stem cells from MTF-1 knockout embryos (42) and KO1-9 cells derived from MTF-1^{-/-} mouse embryo fibroblasts, were grown in Dulbecco modified Eagle medium (GIBCO) supplemented with 5% fetal calf serum (GIBCO) and 5% newborn calf serum (GIBCO), 100 U of penicillin/ml, 100 U of streptomycin/ml, and 2 mM L-glutamine. *Drosophila* S2 cells (48) were grown at 24°C in Schneider's *Drosophila* medium (GIBCO) supplemented with 10% fetal calf serum (Eurobio) and 100 U of penicillin/ml and 100 U of streptomycin/ml. In the case of the transient transfection assay, 60- by 15-mm cell culture plates (Corning) containing 5 ml of medium were used.

Transient transfections and nuclease S1 mapping. Transfections and nuclease S1 mapping of transcripts were performed as described previously (30, 43, 56). Various OVEC reporter constructs driven by either a mammalian or *Drosophila* promoter were transfected with or without respective expression plasmids using the calcium phosphate technique and together with dsRNA when necessary. For heavy metal induction, ZnCl₂, CdCl₂, or CuSO₄ was added to the tissue culture medium to a final concentration as indicated in the figure legends and was incubated for 4 or 24 h before harvesting for mammalian or *Drosophila* S2 cells, respectively. S1 nuclease data were developed using a PhosphorImager (Molecular Dynamics) and quantified using ImageQuant software.

Preparation of nuclear extracts and EMSA. The electrophoretic mobility shift assay (EMSA) was performed as described by Radtke et al. (43). Binding reactions were performed by incubating 25 fmol of end-labeled, 31-bp-long oligonucleotides containing the core MRE consensus sequence (MRE-s), TGCACAC, with nuclear extracts obtained according to the protocol of Schreiber et al. (49). For competition experiments, 5 pmol of unlabeled oligonucleotides was added to the reaction mixture prior to the addition of the extracts. The MRE-s oligonucleotide used for EMSA is as follows: 5'-CGAGGGAGCTCTGCACACGGCCCGAAAAGTG-3' and 3'-TCGAGCTCCCTCGAGACGTGTGCCGGGCTTTTCACAGCT-5'.

Database searches and computer analyses of the sequences. Database homology searches were carried out using the National Center for Biotechnology Information blast server (2). Splice sites and intron/exon boundaries were determined by alignment of the genomic sequence with the dMTF-1 cDNA. Sequence alignments were performed using the CLUSTALX program.

Nucleotide sequence accession number. The GenBank accession numbers are AJ271817 for the dMTF-1 gene and AJ297844 for the cDNA.

RESULTS

Cloning and expression of dMTF-1. Searching through *Drosophila* databases, we found open reading frames related to MTF-1 on EST sequences derived from adult fly heads and from Schneider cells. However, the MTF-1 candidate sequence reconstructed from EST clones lacked the first zinc finger and also lacked any reasonable in-frame start codon. The complete N-terminal region could only be deduced after isolation and analysis of clones from an embryonic cDNA library provided by Fu and Noll (22) (GenBank accession no. AJ297844). The dMTF-1 locus was mapped to (3L) 67B using a digoxigenin-labeled cDNA hybridization probe in a salivary gland chromosome squash preparation (not shown). This chromosome region also contains a number of lethal and nonlethal mutations; however, none of them bears a phenotype that can easily be related to the loss of a putative regulator of heavy metal or other stress. Sequencing of a genomic insert in a lambda phage clone revealed the presence, besides dMTF-1, of three additional genes: the gene for ribosomal protein S17 (*RpS17*), in opposite orientation; the gene for SHC adapter protein (*Shc*), coding for a putative regulator of epidermal growth factor receptor signaling; and the 3' end of the *aay* gene. This gene constellation was confirmed by the *Drosophila* genomic sequence (1). The intron-exon structure of dMTF-1 reveals

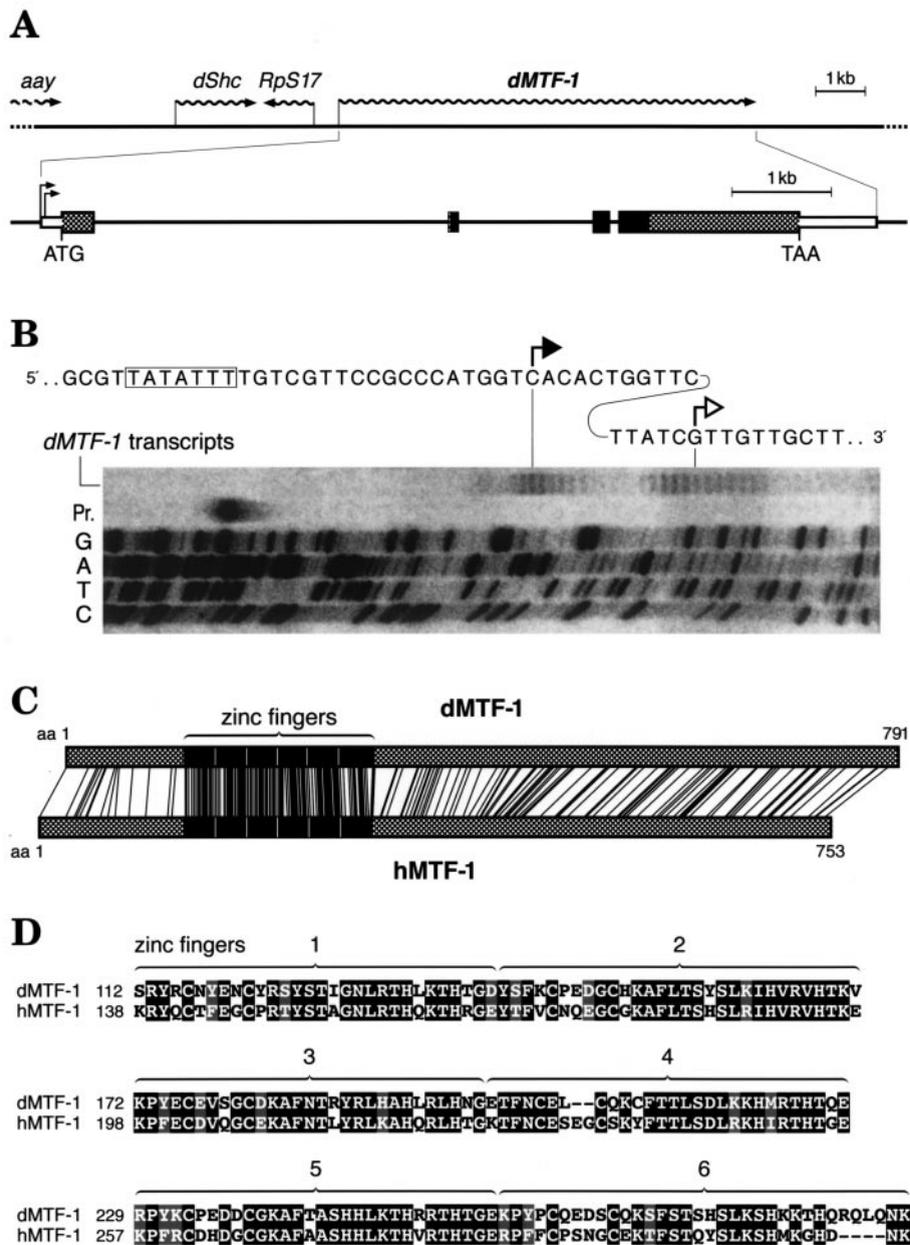


FIG. 1. Structure of the *dMTF-1* locus. (A) Schematic view of the *dMTF-1* locus and neighboring genes (GenBank accession no. AJ271817 for the gene, AJ297844 for cDNA). The *dMTF-1* gene is preceded by the astray (*aay*), SHC adapter protein (*dShc*) (34), and ribosomal protein S17 (*RpS17*) genes. The *dMTF-1* gene contains two large introns and one small intron of 3,571, 1,362, and 108 bp, respectively. The position of intron 3 coincides exactly with that of a vertebrate intron in *MTF-1*. The DNA-binding zinc finger region is indicated by black boxes, and the rest of the coding region is indicated by dotted boxes. Note that in the EST database and in the *Drosophila* Genome Annotation Database, only a cDNA that lacks exon 2 (zinc finger 1) is listed. (B) Mapping of the *dMTF-1* gene transcription start sites. Cytoplasmic total RNA was isolated from *Drosophila* S2 cells after transfection with a *dMTF-1* genomic clone. The RNA was digested with DNase I and then subjected to S1 nuclease mapping after hybridization to a ³²P-labeled DNA oligonucleotide probe corresponding to the promoter region of *dMTF-1* (electrophoresis is shown from left to right). The lane “*dMTF-1* transcripts” shows two clusters of initiation sites downstream of a putative TATA box (framed), which coincide with the RACE extension product (solid arrowhead) and the 5' end of the longest *dMTF-1* cDNA clone (empty arrowhead), respectively. Pr., input oligonucleotide probe; G, A, T, and C, sequencing reaction of the *dMTF-1* promoter region used as reference. (C) Similarity of *dMTF-1* and *hMTF-1* proteins. Identical amino acids (aa) are indicated by connecting lines. The zinc finger regions (black) are best conserved with 66% identical/78% similar amino acids. (D) Alignment of the amino acid sequences in the zinc finger region of *dMTF-1* and *hMTF-1*. Zinc fingers 1 to 6 are indicated by numbered brackets above the corresponding sequences.

three introns; the last one is at a position identical to that for the one in vertebrates (3, 4), while the other two intron positions are unique to *Drosophila* (Fig. 1A) (GenBank accession no. AJ271817). To determine more accurately the transcrip-

tion start site, we performed an extension reaction (RACE) and also determined the 5' end of transcripts independently by transcript mapping with nuclease S1 (Fig. 1B). These data are consistent and indicate a transcription start downstream of a

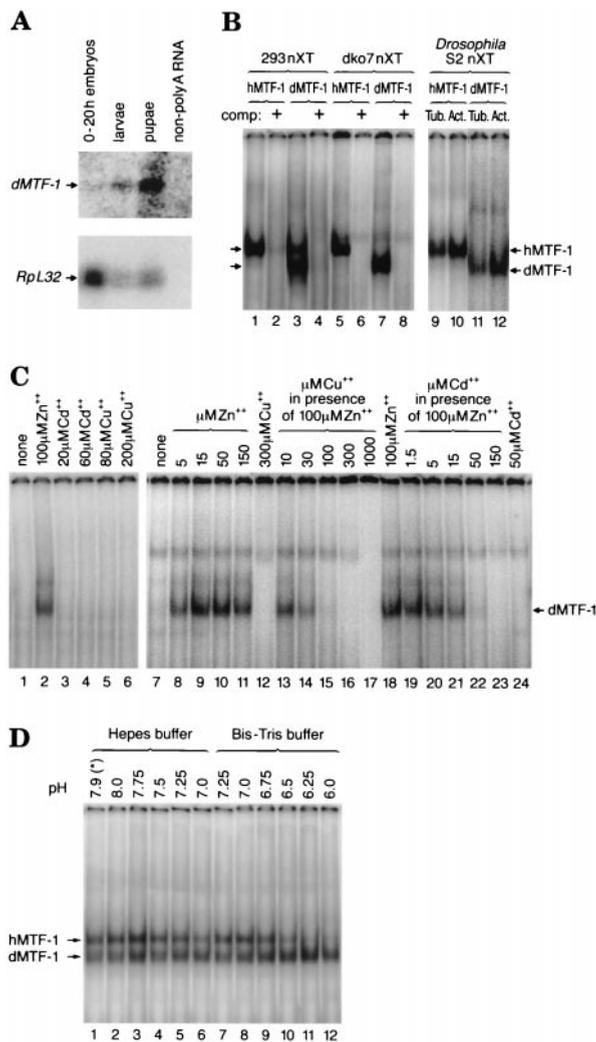


FIG. 2. Expression of dMTF-1 in *Drosophila*. (A) Northern blot with poly(A) RNA of embryos, larvae, and pupae. Poly(A) RNA was isolated, and approximately 5 μ g of the RNA was loaded, electrophoresed, blotted, and hybridized to a cDNA probe corresponding to *dMTF-1* or ribosomal protein L32 (*RpL32*) according to standard procedures. The result shows a steady increase of *dMTF-1* transcripts, following development of *Drosophila* from embryos (0 to 20 h old) to larvae to pupae, relative to those of *RpL32*. Non-poly(A) RNA in the last lane was used as a negative control. (B) EMSA of d- and hMTF-1. All the binding reactions were performed in the presence of 100 μ M ZnCl₂. Human embryonic kidney 293 cells were transfected with hMTF-1 (lanes 1 and 2) or dMTF-1 (lanes 3 and 4) cDNA expression plasmids, and 15 μ g of nuclear protein extract (nXT) was used for bandshift. Lanes 1 and 3, bandshift with ³²P-labeled MRE consensus oligonucleotide (MRE-s). Lanes 2 and 4, same conditions but also including a 200-fold excess of unlabeled MRE-s competitor oligonucleotide (comp +). The upper band in lane 3 is from endogenous hMTF-1. Mutant mouse cells (designated dko7) lacking MTF-1 due to targeted gene disruption were transfected with hMTF-1 (lanes 5 and 6) or with dMTF-1 (lanes 7 and 8) expression plasmids, and 12 μ g of nuclear protein extract (nXT) was used for bandshift. Lanes 5 and 7, presence of labeled MRE-s probe; lanes 6 and 8, presence of 200-fold cold competitor. In the case of *Drosophila* S2 cells transfected with expression clones for hMTF-1 (lanes 9 and 10) or dMTF-1 (lanes 11 and 12), two different constitutive *Drosophila* promoters were used to drive MTF-1 expression, namely, *D. tubulin α 1* (Tub.) (lanes 9 and 11) and *D. actin 5C* (Act.) (lanes 10 and 12). Fifteen micrograms of nuclear protein extract (nXT) was used for bandshift. Note that hMTF-1 migrates unusually slowly compared to the similarly sized dMTF-1;

putative TATA box and a 3.4-kb mRNA for *dMTF-1*. *dMTF-1* cDNA encodes a protein of 791 amino acids (predicted molecular mass, 85 kDa), which is slightly larger than vertebrate MTF-1. A comparison of the protein sequences between the three vertebrate species and *Drosophila* reveals a particularly striking similarity in the region of all six zinc fingers (Fig. 1C and D). The similarity is 78% in the zinc finger region and 27% outside it, i.e., 39% in the total protein. In order to verify the data obtained by sequencing and transcript mapping (Fig. 1B), we also performed a Northern blot with samples of *Drosophila* poly(A) RNA (Fig. 2A), which indicates a steady increase of *dMTF-1* mRNA in embryos, larvae, and pupae relative to the mRNA for ribosomal protein L32. In order to obtain some information on the expression of *dMTF-1* in the adult, frozen tissue sections were subjected to in situ hybridization. Preliminary results indicate a strong expression in the fat body and in the gut (not shown), consistent with a major role in the control of MT (*Mtn*), which is expressed in the gut and also the fat body of the adult fly (8, 9, 19, 35).

dMTF-1 activates MT promoters in vivo. To assess the biological properties of dMTF-1, we cloned the full-length cDNA under the control of either the *D. tubulin α 1* or *D. actin 5C* promoter. After transfection into cultured *Drosophila* S2 cells, nuclear extract preparation, and EMSA, a characteristic band migrating slightly faster than the one with hMTF-1 became apparent (Fig. 2B). dMTF-1 protein could also be readily identified after transfection into mammalian cells (Fig. 2B). As was found with mammalian MTF-1, the binding of dMTF-1 to a consensus MRE was competed by an MRE sequence but not by a GC box oligonucleotide that specifically binds Sp1 factor (Fig. 2B and data not shown). dMTF-1, like mammalian MTF-1, depends on zinc for DNA binding in vitro, and other heavy metals like copper and cadmium cannot replace it but rather interfere with DNA binding (Fig. 2C). Nevertheless, there is also a difference in DNA-binding activity between h- and dMTF-1: when the reaction was performed at different pH values between 6.0 and 8.0, dMTF-1 showed a greater tolerance than did hMTF-1 towards low pH (6.0 to 6.5), perhaps reflecting the low optimum pH (6.5) of *Drosophila* cells (Fig. 2D). We also found that hMTF-1 is not damaged at low pH

this is attributed to the higher proline content of the former, a condition known to retard electrophoretic migration (e.g., reference 42). (C) DNA-binding activity of dMTF-1 is dependent on zinc but not on cadmium or copper. Mouse embryonic stem cells lacking endogenous MTF-1 (dko7 cells) (lanes 1 to 6) or mouse embryo fibroblasts derived from MTF-1 knockout embryos (KO1-9 cells) (lanes 7 to 24) were transfected with dMTF-1 expression plasmids. Nuclear extracts were prepared 40 h later, and 12 μ g (lanes 1 to 6) or 8.5 μ g (lanes 7 to 24) of protein was used for each EMSA. Different amounts of heavy metal ions (ZnCl₂, CdCl₂, or CuCl₂) were included in the binding reactions as indicated in the figure. Arrow, position of MRE-s oligonucleotide bound by dMTF-1. (D) Species-specific difference of MTF-1 DNA binding at low pH. Eighteen micrograms of protein from dMTF-1-transfected human 293 cell nuclear extracts was used in an EMSA with binding buffers of different pH buffered by either HEPES (lanes 1 to 6) or bis-Tris (lanes 7 to 12). ZnCl₂ (100 μ M) was included in the binding reaction. Note that the reactions at pHs 7.25 and 7.0 were performed with each of the two binding buffers, to account for possible differences in buffer properties. The lane marked with an asterisk is with our standard EMSA binding buffer, i.e., pH 7.9 buffered by HEPES.

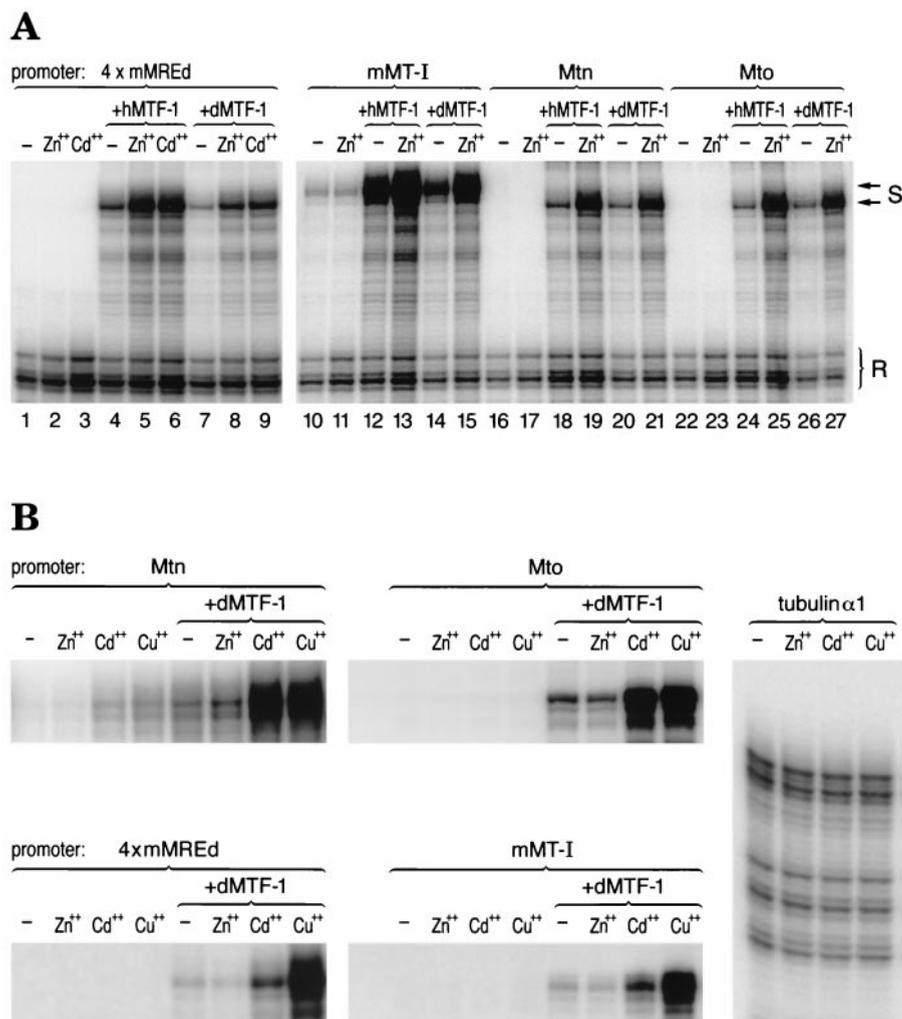


FIG. 3. Transcriptional activity of dMTF-1 in mammalian and *Drosophila* cells. (A) Comparison between the activity of hMTF-1 and dMTF-1 on both mammalian (mMT-I, 4× mMREd) and *Drosophila* (*Mtn*, *Mto*) MT promoters in a mammalian cell line lacking endogenous MTF-1 (dko7). Reporter gene constructs containing MT promoters were transfected into dko7 cells together with a reference gene construct driven by the CMV promoter, with or without cotransfection of an expression vector for hMTF-1 or dMTF-1. Cytoplasmic total RNA was extracted after metal induction for 4 h and was subjected to nuclease S1 mapping. With the mMT-I promoter (lanes 10 to 15) and the synthetic promoter 4× mMREd (lanes 1 to 9), dMTF-1 conferred metal-inducible transcription, though not as efficiently as hMTF-1 (compare lanes 12 and 14 and 4 and 7 for basal activation; lanes 13 and 15 and 5 and 8 for Zn²⁺ response; and lanes 6 and 9 for Cd²⁺ response). The *Drosophila* MT promoters (*Mtn* and *Mto*) responded equally well to both hMTF-1 and dMTF-1 in the same experiment. (In the case of the *Mtn* promoter, compare lanes 18 and 20 for basal activation and lanes 19 and 21 for Zn²⁺ induction. In the case of the *Mto* promoter, compare lanes 24 and 26 for basal activity and lanes 25 and 27 for Zn²⁺ induction.) Note that in all the cases, the CMV promoter was not affected by either cotransfection of MTF-1 (hMTF-1 or dMTF-1) or metal induction. Zn²⁺ induction, 100 μM ZnCl₂; Cd²⁺ induction, 50 μM CdCl₂; S, reporter gene signal (MT promoters); R, reference gene signal as an internal control (CMV promoter); -, control. (B) *Drosophila* *Mto* and *Mtn* promoters as well as mammalian MT (mMT-I, 4× mMREd) promoters are active in *Drosophila* S2 cells in response to heavy metals and dMTF-1. OVEC reporter gene constructs driven by the corresponding testing promoters were transfected into S2 cells; where indicated, a dMTF-1 expression vector under the control of the *D. actin 5C* promoter was cotransfected. Cytoplasmic total RNA was isolated after heavy metal induction for 24 h, and 160 μg of the RNA was subjected to nuclease S1 mapping. As a control, heavy metal treatment did not have any effect on the *D. tubulin α1* promoter. Zn²⁺ induction, 100 μM ZnCl₂ for mMT-I and *Mto*; 200 μM for 4× mMREd; 500 μM for *Mtn* and *tubulin α1*. Cd²⁺ induction, 20 μM CdCl₂. Cu²⁺ induction, 500 μM CuSO₄. -, control.

(6.0 at room temperature for 1 h), since binding could be fully restored upon shift to high pH (not shown).

To test for biological activity of dMTF-1, it was first compared side by side with hMTF-1 in a transient transfection assay in a mammalian cell line which was derived from mouse embryonic stem cells lacking endogenous MTF-1 (dko7 cells) (Fig. 3A). In that comparison dMTF-1 showed a very similar, though somewhat less efficient, response to zinc and cadmium

induction than did hMTF-1. As a control, the viral CMV promoter was not affected by heavy metal treatment and/or expression of extra MTF-1. Similar results were also obtained with dMTF-1 when transfected into another cell line derived from MTF-1^{-/-} mouse embryo fibroblasts (designated KO 1-9 cells) (data not shown). For a test in fly cells, dMTF-1 cDNA driven by *D. actin 5C* promoter was transfected, together with reporter genes, into S2 cells. As shown in Fig. 3B and 4,

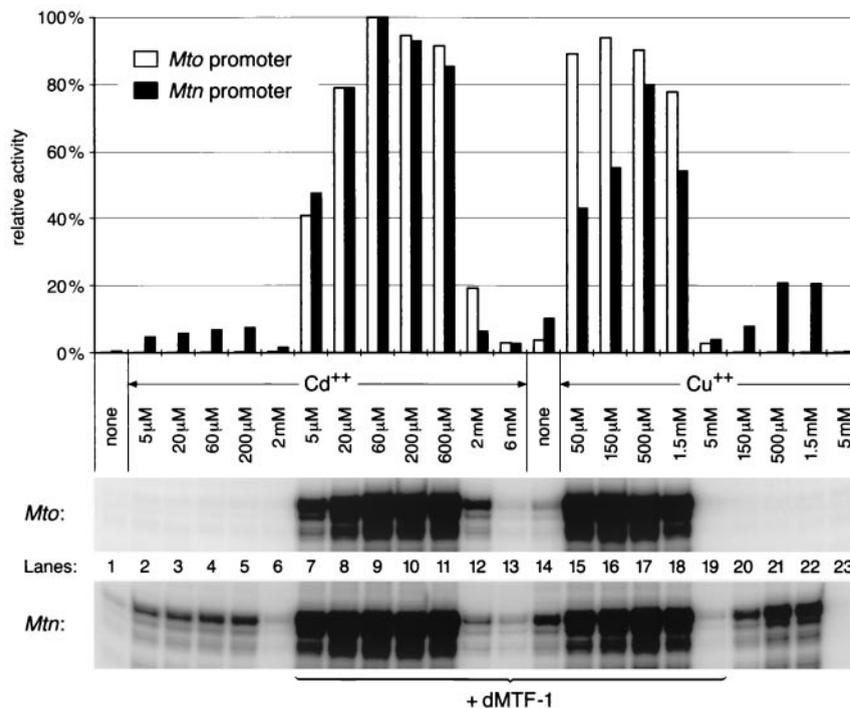


FIG. 4. Induction of *Drosophila* MT *Mto* and *Mtn* promoters by dMTF-1 in response to different heavy metal concentrations. Reporter gene constructs driven by the promoters of *Drosophila* MT genes (*Mto* and *Mtn*) were transfected into *Drosophila* S2 cells. Induction by cadmium or copper was tested with different heavy metal concentrations for 24 h, with and without cotransfected dMTF-1 expression vector. To determine the activity of the *Mto* promoter, 100 μ g of cytoplasmic RNA was used except for lanes 13 (50 μ g), 19 (25 μ g), and 23 (25 μ g), where a fraction of cells was killed by high metal concentration. For the *Mtn* promoter, 160 μ g of RNA was used except for lanes 6 (150 μ g), 12 (100 μ g), 13 (100 μ g), 19 (40 μ g), and 23 (40 μ g) due to the same reason as above. Shown above the autoradiograms is the quantification, in which *Mto* and *Mtn* are represented and signals are normalized for total RNA content. Note that the TATA-less *Mto* promoter (top row of gel) has weak activity in S2 cells and even with cotransfected dMTF-1 expression vector has low basal activity; upon metal load, its activity is similar to that of the *Mtn* promoter.

dMTF-1 is able to confer very strong transcriptional activity to both *Drosophila* MT promoters (*Mto* and *Mtn*) in response to both copper and cadmium but hardly, if at all, to zinc even at a 500 μ M concentration. A clear response of the *Mtn* promoter was achieved, however, when the cells were exposed to 2 mM zinc (Fig. 6A). Unfortunately, dMTF-1 and hMTF-1 could not be studied side by side in *Drosophila*, since transfected hMTF-1 was transcriptionally inactive in S2 cells (not shown; see also Discussion). As a control, the *D. tubulin* α 1 promoter was neither affected by heavy metal treatment nor by expression of transfected dMTF-1 (Fig. 3B and data not shown).

S2 cells contain only low amounts of dMTF-1 (not shown) unless transfected with a dMTF-1 expression vector. Accordingly, the TATA-less *Mto* promoter was hardly active in S2 cells but became strongly metal-inducible in the presence of transfected dMTF-1. Even the adult-type *Mtn* promoter, while responsive to heavy metals on its own, depended on extra transfected dMTF-1 for maximal activity (Fig. 3B and 4). Comparable results were obtained with *lacZ* as a reporter gene under the control of either *Mto* or *Mtn* promoter when transfected together with dMTF-1 expression vector (data not shown), which is consistent with previous findings that MT levels in *Drosophila* are primarily regulated at the level of transcription (9, 10, 35, 39) and suggests that dMTF-1 plays a critical role in heavy metal homeostasis.

dMTF-1 activates transcription via MRE sequence motifs.

To determine whether the four putative MREs of the embryonic *Mto* promoter were indeed responsive to heavy metals, we made a synthetic minipromoter which merely contained these MRE motifs in a tandem array (Fig. 5). The activity of this promoter shows that the *Drosophila* MREs alone are sufficient to confer heavy metal inducibility to a corresponding reporter gene.

RNAi demonstrates dependence of MT promoters on dMTF-1. To further analyze the role of dMTF-1 in the transcriptional regulation of MT genes, we also applied the technique of dsRNA-mediated interference (RNAi) (21, 24, 31). A 512-bp segment of the *dMTF-1* cDNA was flanked on either side by a T7 phage promoter and transcribed in a cell-free reaction by T7 RNA polymerase. The dsRNA was purified by phenol extraction followed by ethanol precipitation, and various amounts of dsRNA were cotransfected with a constant amount of reporter gene. As seen in Fig. 6A, RNAi eliminated all transcripts from a reporter gene with an MT (*Mtn*) promoter that was driven by the *Drosophila* host cell's transcription factors, indicating that MT transcription depends on dMTF-1. dsRNA interference was highly effective: even when dMTF-1 was overexpressed from a cotransfected gene, there was still a strong inhibition of reporter gene transcription (Fig. 6B). The transfected *Mtn* as well as the *Mto* promoter con-

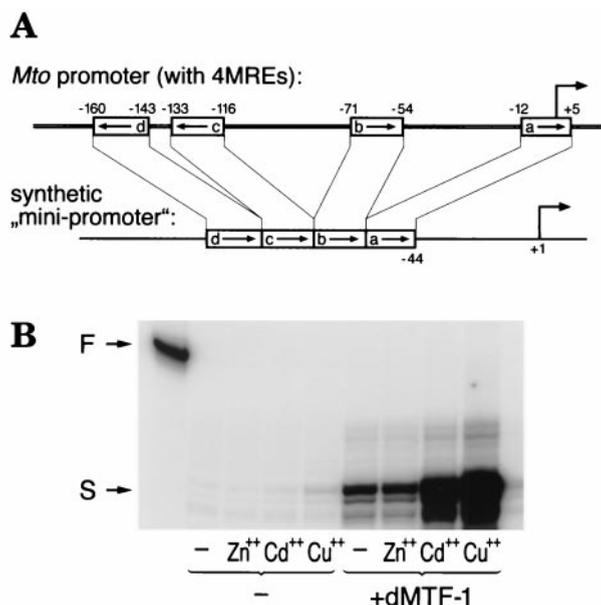


FIG. 5. *Drosophila* MRE-like motifs can confer heavy metal response. The promoter of the embryonic MT gene (*Mto*) of *Drosophila* contains four MRE-like motifs (51), which, however, were found by EMSA to bind relatively poorly to MTF-1 under our standard experimental conditions. (A) To evaluate their potential, a minipromoter was assembled into a OVEC reporter construct as shown (for details, see Materials and Methods) and was transfected into S2 cells with or without a dMTF-1 expression vector. a to d indicate four MRE-like sequence motifs. Numbering refers to nucleotide position relative to transcription start. (B) Cytoplasmic RNA was isolated after metal induction for 24 h, and 160 μ g of the RNA was subjected to nuclease S1 mapping as shown. Zn²⁺ induction, 100 μ M ZnCl₂; Cd²⁺ induction, 20 μ M CdCl₂; Cu²⁺ induction, 500 μ M CuSO₄; F, free S1 oligonucleotide probe; S, correctly initiated reporter gene transcripts. -, absence of dMTF-1.

structs were inhibited, suggesting that both MT genes rely on MTF-1 for transcriptional induction. Furthermore, the inhibition was specific: dsRNA of the *lacZ* gene blocked β -galactosidase activity (not shown) but failed to block reporter genes with *Mtn* or *Mto* promoters (Fig. 6A and B), and transcripts from the *D. tubulin* α 1 gene promoter were not inhibited by either of the two dsRNAs (not shown). Quite contrary to the specific RNAi effect, transfection of an unrelated dsRNA even boosted the signals, perhaps due to an inhibition of cellular nucleases by excess substrate (Fig. 6A). In agreement with such an interpretation, nonspecific stimulation was also observed with cotransfected yeast tRNA (not shown). Finally, we found that *dMTF-1* dsRNA inhibited not only the activation of a transfected MT promoter but also significantly reduced the transcripts from the endogenous MT *Mtn* gene (Fig. 6C). Under standard transfection conditions, not all cells can be transfected; thus, our finding that the transfected dsRNA reduced (but did not eliminate) the signal with cadmium and copper, while *lacZ* dsRNA was ineffective, is yet more evidence for the role of dMTF-1 in MT gene regulation.

DISCUSSION

In *Drosophila*, the two genes encoding MTs, *Mto* and *Mtn*, have distinct but partially overlapping expression patterns, with

Mto being primarily expressed in early embryogenesis and *Mtn* being expressed in late embryogenesis/adulthood. In addition, *Mtn* is expressed in hemocytes, possibly to regulate copper supply to hemocyanin (8). It was postulated that *Drosophila Mto* is important for copper homeostasis during embryogenesis, while *Mtn*, in particular due to its very strong expression in the gut, Malpighian tubules, and fat body, is thought to balance the toxic effects of copper and other metals, such as cadmium and mercury. Thus, *Mtn* seems to play a role that in the snail is delegated to two different MTs, one active in hemocytes, the other one in the gut (14).

Here we have isolated a cDNA and dMTF-1, the corresponding gene from *Drosophila* encoding a transcription factor related to vertebrate MTF-1. The expression pattern of dMTF-1 is compatible with a role in MT gene activation/heavy metal detoxification and homeostasis. Furthermore, in transfection experiments both in mammalian cells and *Drosophila* S2 cells, dMTF-1 is able to confer strong cadmium induction to MT promoters of both *Drosophila* and mammalian origins. As in mammals, metal induction seems to involve characteristic MRE sequence motifs. In support of this notion, the promoters of both *Drosophila* MT genes, *Mto* and *Mtn*, contain multiple sequence motifs resembling mammalian MREs. Their role in metal response was further corroborated by a synthetic minipromoter where the four MREs from the *Mto* gene were juxtaposed in a tandem array and conferred strong heavy metal response to the reporter gene (Fig. 5). In spite of some striking similarities, it should also be pointed out that significant differences exist between dMTF-1 and vertebrate MTF-1. First of all, the strongly conserved zinc finger domain notwithstanding, there is limited, albeit significant, protein sequence similarity between vertebrate MTF-1 and dMTF-1. Specifically, the three hallmark activation domains of mammalian MTF-1, namely, acidic, proline-rich, and serine/threonine-rich, do not have obvious counterparts in *Drosophila*. The functional equivalents of one or all of them remain to be identified. Secondly, dMTF-1 is quite forgiving towards low pH (6.0 to 6.5) while mammalian MTF-1 loses its DNA-binding capacity under these conditions (Fig. 2D). This property may explain why mammalian MTF-1 in our hands was inactive in transfected *Drosophila* Schneider cells, which are grown at pH 6.5 (not shown). Conversely, dMTF-1 performed well in mammalian cells grown in Dulbecco modified Eagle medium at pH 7.4 (Fig. 3A). A third difference may concern the tissue distribution: while mammalian MTF-1 is expressed in all tissues analyzed so far (4), *dMTF-1* is expressed in a few tissues, notably gut and fat body, although further experiments will have to probe into this issue. This is compatible with a major role in the activation of MT and possibly other toxicity/cell stress-related genes. Finally, there is a difference between mammals and *Drosophila* concerning heavy metal metabolism. In mammalian cells, MTF-1 mediates MT gene transcription primarily in response to zinc and cadmium and, to a lesser extent, to copper and nickel (25). We were surprised to find that dMTF-1 was unable to induce transcription in response to zinc at concentrations which readily induce transcription in mammalian cells. However, at 2 mM zinc, a concentration that is lethal to mammalian cells, dMTF-1 activated transcription from the *Mtn* promoter (Fig. 6A). This finding is in agreement with earlier heavy metal studies of the fly: of all metals tested for *Mto* and *Mtn* tran-

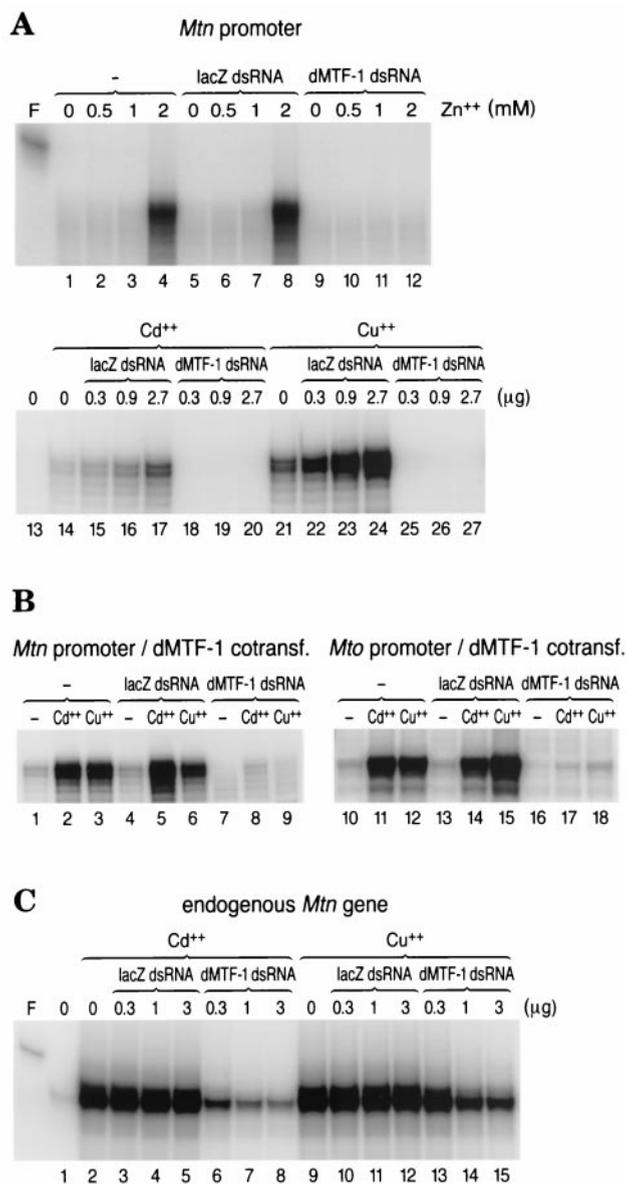


FIG. 6. Inhibition of MT *Mtn* and *Mto* transcription by RNAi specific for *dMTF-1*. (A) dsRNA of *dMTF-1* inhibits heavy metal-induced transcription of the *Mtn* reporter gene. The *Mtn* promoter-reporter gene construct was transfected into *Drosophila* S2 cells, and various amounts (ranging from 0.3 to 2.7 μg per 5 ml of culture medium) of dsRNA of either *dMTF-1* or *lacZ* were included. Cytoplasmic RNA was isolated after 24 h of heavy metal induction (0.5 to 2 mM ZnCl₂ as indicated, 60 μM CdCl₂, or 500 μM CuSO₄) and the reporter gene transcripts were quantified by S1 nuclease mapping. Lanes 1 and 13, basal expression level of transfected *Mtn* reporter; lanes 2 to 4, 6 to 8, and 10 to 12, induction by zinc; lanes 14 to 20, induction by cadmium; lanes 21 to 27, induction by copper. *lacZ* dsRNA was included in lanes 5 to 8, 15 to 17, and 22 to 24 (lanes 5 to 8, 1 μg; lanes 15 to 17 and 22 to 24, 0.3 to 2.7 μg as indicated). *dMTF-1* dsRNA was added in the transfections shown in lanes 9 to 12, 18 to 20, and 25 to 27 (lanes 9 to 12, 1 μg; lanes 18 to 20 and 25 to 27, 0.3 to 2.7 μg as indicated). -, control. F, free probe. (B) Inhibition of heavy metal (Cd²⁺ or Cu²⁺) induction in the presence of cotransfected (cotransf.) *dMTF-1* by dsRNA of *dMTF-1*. The *Mtn* or *Mto* reporter construct was introduced into S2 cells together with a *dMTF-1* expression vector, and results very similar to those for *Mtn* reporter transfection alone (described for panel A) were obtained. Lanes 1 and 10, basal transcription of the *Mtn* and *Mto* reporters, respectively. Lanes 2, 5, 8, 11, 14, and 17, cadmium

induction, Cd²⁺, Cu²⁺, and Hg²⁺ were found to be more efficient than Zn²⁺, which was required at concentrations of at least 1 mM (7, 9, 19, 35, 51). The high concentrations of zinc (and copper) tolerated by Schneider cells may reflect inefficient uptake or, more likely, particularly efficient export of these two essential heavy metals (13, 40). In fact, a search of the *Drosophila* genome revealed six homologs of mammalian zinc transporters (1). Whatever the reason for the poor response to zinc at submillimolar concentrations, it cannot be attributed to a species difference between MTF-1 factors themselves, since *dMTF-1*, upon transfection into mouse cells, mediates zinc-responsive transcription like hMTF-1 (Fig. 3A). Further evidence for functional similarity is that *dMTF-1*, like mammalian MTF-1, requires elevated zinc concentrations for DNA binding in vitro, while copper and cadmium interfere with zinc rather than replacing it (Fig. 2C) (6, 11, 25). At first sight, cadmium and copper induction of a transcription factor that strictly requires zinc appears paradoxical. However, we have recently been able to induce MTF-1-dependent transcription in vitro by cadmium and copper. Activation was dependent on the presence of MT, which has a particularly high affinity for these two metals and upon binding to them releases zinc on behalf of MTF-1 (B. Zhang, O. Georgiev, and W. Schaffner, unpublished). Another question concerns the regulation of the *dMTF-1* gene itself. In mammals, the MTF-1 promoter does not contain MREs and transcripts are marginally, if at all, elevated by heavy metal treatment (4, 25). Although we have not quantified MTF-1 transcripts in *Drosophila*, we note that the MTF-1 promoter, like the mammalian one, lacks MRE-type sequence motifs, at least within a 2-kb segment around the site of transcription initiation.

Perhaps the best evidence for an important role of *dMTF-1* in MT gene regulation is provided by our studies with RNAi. Without RNAi and in the absence of any *Drosophila* mutation in the *dMTF-1* gene, one might have argued that the observed effect in transfected cells was due to a nonphysiological stimulation by a heterologous zinc finger factor. Ectopic activation effects have been observed before, when related family members of mammalian transcription factors could replace each other in transient transfection/overexpression conditions. This scenario is ruled out by the finding that *dMTF-1* dsRNA completely blocked the activation of the *Mtn* promoter without any cotransfected MTF-1 expression plasmid, i.e., under conditions

induction (60 μM CdCl₂, 24 h); lanes 3, 6, 9, 12, 15, and 18, copper induction (500 μM CuSO₄, 24 h). Lanes 4 to 6 and 7 to 9, cotransfection with 10 μg of *lacZ* dsRNA and *dMTF-1* dsRNA, respectively; lanes 13 to 15 and 16 to 18, cotransfection with 5 μg of *lacZ* dsRNA and *dMTF-1* dsRNA, respectively. -, control. (C) dsRNA of *dMTF-1* inhibits endogenous MT gene (*Mtn*) transcription. Various amounts of dsRNA were transfected into S2 cells. Endogenous *Mtn* transcripts were detected by S1 mapping after 24 h of induction with 60 μM CdCl₂ or 500 μM CuSO₄. F, free S1 probe for detecting endogenous *Drosophila* *Mtn* transcripts. Lanes: 1, basal level of endogenous *Mtn* transcripts; 2 and 9, after Cd²⁺ and Cu²⁺ induction, respectively; 3 to 5 and 6 to 8, effects on cadmium induction of dsRNA corresponding to *lacZ* and *dMTF-1*, respectively; 10 to 12 and 13 to 15, effects on copper induction of dsRNA corresponding to *lacZ* and *dMTF-1*, respectively. Endogenous *Mtn* transcripts following Cd²⁺ or Cu²⁺ induction were reduced to 20 and 47%, respectively, of the control level in the presence of *dMTF-1* dsRNA, whereas *lacZ* dsRNA was not inhibitory.

that relied entirely on the host cell's transcription factors. Furthermore, unlike many other transcription factors, MTF-1 is a unique protein without related family members in *Drosophila* and, apparently, in mammals. Taken together, the RNAi experiments corroborated the important role of dMTF-1 for MT transcription and thus heavy metal homeostasis.

It certainly will be of interest to study the effect of loss of *dMTF-1* in vivo, for example, by screening deletion mutants at the *MTF-1* locus or by the newly introduced techniques of inheritable RNAi (32) or targeted gene disruption in *Drosophila* (45). As mentioned, a targeted disruption of MTF-1 in the mouse results in embryonic lethality due to liver decay on embryonic days 13 to 14. We note that disruption of either of two other stress-associated transcription factors, namely, c-Jun (26) and NF- κ B/RelA (5), also results in embryonic death from liver decay at about the same stage of mouse embryogenesis. In this context it is of interest that AP-1 sites, which bind *jun-fos* heterodimers, are present in the promoters of both *Drosophila* MTs, pointing to a possible interconnection of MTF-1 and AP-1 in the cellular stress response of insects. Thanks to the availability of *jun* (27, 33) and *fos* (1, 44, 60) mutants and the power of *Drosophila* genetics, these and other aspects of cellular stress response are amenable to analysis.

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