The transcription factors MTF-1 and USF1 cooperate to regulate mouse metallothionein-I expression in response to the essential metal zinc in visceral endoderm cells during early development

Glen K. Andrews^{1,2}, Dae Kee Lee^{1,3},
Rudravajhala Ravindra¹, Peter Lichtlen^{4,5},
Mario Sirito⁶, Michele Sawadogo⁶ and
Walter Schaffner⁵

¹Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KN 66160-7421, ⁶Department of Molecular Genetics, University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA and ⁵Institute for Molecular Biology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

³Present address: Department of Genetics, Lineberger Cancer Center, Room 11-109, Campus Box 7264, University of North Carolina, Chapel Hill, NC 27599-7264, USA

⁴Present address: ESBATech AG, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

²Corresponding author
e-mail: gandrews@kumc.edu

During early development of the mouse embryo, expression of the metallothionein-I (*MT-I*) gene is heightened specifically in the endoderm cells of the visceral yolk sac. The mechanisms of regulation of this cell-specific pattern of expression of metallothionein-I are unknown. However, it has recently been shown that MTF-1, functioning as a metalloreulatory transcription factor, activates metallothionein genes in response to the essential metal zinc. In contrast with the metallothionein genes, *MTF-1* is essential for development; null mutant embryos die due to liver degeneration. We report here that *MTF-1* is absolutely essential for upregulation of *MT-I* gene expression in visceral endoderm cells and that optimal expression also involves interactions of the basic helix–loop–helix upstream stimulatory factor-1 (USF1) with an E-box1-containing sequence at –223 bp in the *MT-I* promoter. Expression of *MT-I* in visceral endoderm cells was dependent on maternal dietary zinc. Thus, the essential metal, zinc, apparently provides the signaling ligand that activates cell-specific *MT-I* expression in visceral endoderm cells.

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Introduction

The metallothioneins (MTs) are a family of metal-binding proteins that have been conserved during evolution. In mice, four *MT* genes have been identified (Palmiter *et al.*, 1993a). *MT-I* and *-II* are coordinately expressed in many cell types and are highly inducible (Searle *et al.*, 1984). In contrast, *MT-III* expression has been detected only in subpopulations of cells in the brain (Palmiter *et al.*, 1993a), the maternal deciduum (Liang *et al.*, 1996) and several reproductive tissues (Moffatt and Seguin, 1998), and *MT-IV* expression has been detected in subpopulations

of cells in stratified squamous epithelia (Palmiter *et al.*, 1993a) and in the maternal deciduum (Liang *et al.*, 1996). Neither *MT-III* nor *MT-IV* appears to be induced by agents (i.e. zinc and cadmium) that induce *MT-I* and *-II* (Palmiter *et al.*, 1993a; Liang *et al.*, 1996; Moffatt and Seguin, 1998).

Distinct cell-specific activation of mouse *MT-I/II* also occurs during embryonic development (Andrews *et al.*, 1987a, 1993; Karasawa *et al.*, 1991). Initially, *MT-I* and *-II* are highly expressed specifically in the endoderm of the yolk sac (Andrews *et al.*, 1984). Visceral endoderm cells are the third cell type to differentiate from the inner cell mass (Gardner, 1982). Together with a single inner layer of mesoderm cells, they form the visceral yolk sac, which surrounds the developing embryo until late in pregnancy (Padykula *et al.*, 1966). Visceral endoderm cells form a secretory epithelium responsible for the synthesis of the major serum and amniotic fluid proteins, such as α -fetoprotein and transferrin (Dziadek and Adamson, 1978; Janzen *et al.*, 1982). The visceral yolk sac is also the first site of hematopoiesis (De la Chapelle *et al.*, 1969) and plays a role in transferring maternal nutrients (e.g. amino acids and vitamins) and proteins (e.g. IgG) into the embryonic environment (Padykula *et al.*, 1966).

There is no definitive information on the mechanisms that regulate the cell-specific activity of *MT*. However, many different agents and stresses can induce *MT* genes (Miles *et al.*, 2000) and the mouse *MT-I* proximal promoter is structurally complex (Stuart *et al.*, 1985). Metal ion regulation of *MT* expression is mediated by metal response elements (MREs). MRE-binding transcription factor-1 (MTF-1), a six zinc-finger protein of the Cys₂His₂ family, binds directly and specifically to MREs (Westin and Schaffner, 1988; Radtke *et al.*, 1993; Koizumi *et al.*, 1999) and regulates metal-induced and basal *MT* expression in cultured cells (Heuchel *et al.*, 1994). MTF-1 is thought to function as a specific sensor of free cytoplasmic zinc (for review see Andrews, 2000). The mouse *MT-I* promoter contains five MREs located in the proximal 250 bp (Stuart *et al.*, 1985) and binding sites for the transcription factors upstream stimulatory factor (USF), Sp1, Nrf2 (Dalton *et al.*, 1994; Venugopal and Jaiswal, 1996; Li *et al.*, 1998) and perhaps CCAAT/enhancer binding protein (C/EBP)-like proteins (Aniskovitch and Jacob, 1997).

Several transcription factors modulate *MT-I* gene expression (Carthew *et al.*, 1987; Dalton *et al.*, 1994; Li *et al.*, 1998). Of particular importance here is USF1. USFs are basic helix–loop–helix (bHLH)–Zip proteins that form DNA-binding homodimers and heterodimers (Gregor *et al.*, 1990; Sirito *et al.*, 1992; Viollet *et al.*, 1996); two USF genes (*USF1* and *USF2*) have been described (Gregor *et al.*, 1990; Sirito *et al.*, 1994). The proximal promoter of the mouse *MT-I* gene has two binding sites for USF

(Carthew *et al.*, 1987; Li *et al.*, 1998). One site overlaps an antioxidant response element (ARE; Dalton *et al.*, 1994). This composite element (USF/ARE) participates in oxidant induction of *MT* gene expression by stress and cadmium (Dalton *et al.*, 1994; Li *et al.*, 1998) and can regulate basal expression of the gene in cultured cells (Carthew *et al.*, 1987; Dalton *et al.*, 1994). The second and higher-affinity USF-binding site is an E-box (E-box1) sequence CACATG (Li *et al.*, 1998), but no function has been ascribed to this site.

The present study provides the first molecular explanation for a cell-specific pattern of expression of mouse *MT-I* during early development. Unexpectedly, it was found that *MTF-1* is absolutely essential for expression of *MT-I* in the endoderm cells of the visceral yolk sac. Remarkably, the essential metal, zinc, appears to provide the signaling ligand for activation of this gene; this, in turn, suggests that zinc is not equally distributed and/or utilized among tissues. We also report the novel finding that *MTF-1* functions in cooperation with *USF1* (and perhaps other factors), which interact with the E-box1 sequence noted above, to direct this cell-specific pattern of *MT-I* expression.

Results

Visceral endoderm-specific *MT-I* expression requires the proximal promoter in transgenic embryos

A series of mutations in the *MT-I* promoter was examined for the ability to direct cell-specific expression of the *hPAP* reporter in transgenic mouse embryos (Figure 1A). *hPAP* encodes an alkaline phosphatase that, unlike endogenous mouse PAP, is heat-stable. Microinjected eggs were transferred into the oviduct of recipient females; on day 14 of pregnancy, embryos were harvested for PCR genotype determination and the visceral yolk sac/placenta was fixed and analyzed by histochemical staining of hPAP. Only tissues from transgenic embryos contained detectable hPAP staining.

The *MT-I* promoter (742 bp) directed active expression of *hPAP* specifically in the endoderm cells of the visceral yolk sac (Figure 1B; insert). In contrast, only a few cells in the placenta or associated membranes were weakly stained (Figure 1B). Visceral endoderm expression was dependent on a 108 bp region (bases -43 to -150 inclusive) of the proximal 742 bp of the promoter (Figure 1; compare -742PAP with -742 δ PAP) (Figure 1B). This 108 bp region in -153PAP did not exhibit strong activity alone, and hPAP staining was mosaic and weak in the visceral yolk sac (not shown). This suggests that promoter elements cooperate to direct expression in the visceral endoderm.

MT-I expression requires multiple promoter elements in transgenic embryos

Deletion analysis of the promoter demonstrated that the proximal 250 bp exhibited strong, endoderm-specific activity (Figure 1). A high-affinity binding site for USF is located at -223 bp in this promoter (Li *et al.*, 1998), so we investigated whether this element was involved in cooperation with the proximal 153 bp. Addition of this element to the proximal -153 bp promoter restored high-

Fig. 1. Elements in the proximal promoter of mouse *MT-I* cooperate to direct high level *hPAP* reporter expression in the visceral yolk sac. (A) Diagrammatic representation of the mouse *MT-I* promoter-*hPAP* fusion constructs. The *MT-I* promoter provided the transcription start point (+1, straight arrow) and +66 bp of untranslated region, in addition to the base pairs of upstream promoter as indicated (Dalton *et al.*, 1994). *hPAP* provided the translation start codon and complete coding sequence, and contains several introns (Knoll *et al.*, 1988). A second polyadenylation signal is placed downstream of *hPAP* (Lin and Culp, 1991). Elements in the -742 bp promoter are as follows: E-box2 and E-box1, CAnnTG (Liang *et al.*, 1996; Li *et al.*, 1998); Sp1 GC-box (Briggs *et al.*, 1986); MREs a-d, metal response elements (Stuart *et al.*, 1984); USF/ARE, USF-binding site (not an E-box) overlapping an antioxidant response element (Dalton *et al.*, 1994); TATA, TATA box. The -742 δ promoter has a 108 bp region (bases -43 to -150 inclusive) deleted. The E-box1:-153 promoter has a single, forward-oriented copy of E-box1 inserted just upstream of the -153 bp *MT-I* promoter. *hPAP*, human placental alkaline phosphatase reporter; VYS, visceral yolk sac. The pattern of staining in the visceral yolk sac was assessed qualitatively [see (B)]: +++, staining was always very dark and throughout the yolk sac endoderm; -, no cells stained in the visceral yolk sac in any transgenic embryos; ++, staining was dark in most cells; +, staining was weak and mosaic in the visceral yolk sac and detected in only one embryo. *Number of F₀ transgenic conceptuses that expressed the *hPAP* gene/total number of transgenic embryos detected by PCR. (B) Histochemical detection of hPAP in F₀ transgenic embryos. Promoter constructs are described above. VYS, visceral yolk sac; PL, placenta. Whole mounts of placentae were cross-sectioned before fixation and staining. The insert by the -742PAP whole mount is a photomicrograph of a thin section of the visceral yolk sac that shows *hPAP* expression in visceral endoderm ('endo') cells, but not in mesodermal cells ('meso').

Fig. 2. Visceral endoderm cell nuclei contain USF1 and USF2, which can bind *in vitro* to E-box1. (A) Nuclear proteins were prepared from day 13 visceral yolk sac, placenta and fetal liver, as well as from the maternal liver and pancreas. Nuclear proteins were analyzed by EMSA (Dalton *et al.*, 1996c, 1997; Li *et al.*, 1998) for E-box1-binding and for Sp1- and MTF-1-binding activities (data not shown). The E-box1-binding complex (*USF1/2*) contained USF1 and USF2 and was dependent on the E-box core-binding sequence (data not shown), as described (Li *et al.*, 1998; Sirito *et al.*, 1998). (B) Visceral yolk sacs and placentae were fixed and paraffin sections were analyzed for USF1 and USF2 proteins using immunohistochemistry. Nuclear staining was detected in the visceral yolk sac and placenta.

level expression of *hPAP* in the visceral endoderm in two of the four transgenic embryos examined (Figure 1B). Although a limited number of embryos was examined, these results suggest a role for this element in endoderm-specific *MT-I* gene expression.

E-box1-binding activity in the visceral yolk sac was examined by electrophoretic mobility shift assay (EMSA; Figure 2A). The E-box1-binding complex detected was similar in all tissues examined and was indistinguishable from that obtained in cultured mouse cells (Li *et al.*, 1998). Competition EMSA and supershift assays using antisera against USF1 and USF2 confirmed that this binding activity involves USF1 and USF2 and requires the E-box core sequence (data not shown; see Li *et al.*, 1998). Immunohistochemical localization of USF1 and USF2 also confirmed the presence of these proteins in the nuclei of visceral endoderm cells (Figure 2B). Sp1 (Figure 3B) and MTF-1 DNA-binding activities were also detected in nuclear extracts from these cells (data not shown).

Visceral endoderm-specific *MT-I* expression is dependent on *MTF-1* and *USF1*

The multiple MREs in the *MT-I* promoter are within the 108 bp region that is essential for expression in the visceral yolk sac. This suggested that MTF-1 may play a role in transcriptional activation of this gene in endoderm cells. *MTF-1* knockout mice were used to test this possibility. Although homozygous *MTF-1*^{-/-} knockout embryos die on day 14, they develop normally until then. Heterozygous *MTF-1* knockout (*MTF-1*^{+/-}) mice were inbred and the embryos were examined at day 12 of pregnancy. The genotype of each embryo was determined by PCR and MT protein was immunolocalized in the visceral yolk sac/placenta (Figure 3A). MT immunostaining was strong in the visceral endoderm from normal and heterozygous

embryos, but was absent from the visceral endoderm of all of the homozygous embryos.

The loss of MT protein from the visceral endoderm of the homozygous *MTF-1* knockout embryos was specific (Figure 3B). Immunohistochemical detection of Sp1, another zinc-finger transcription factor, was unaffected, as was immunostaining for α -fetoprotein, the major visceral endoderm secretory protein (Figure 3B). In addition, MT-immunoreactive cells in the outer placenta, probably decidua basalis cells, were detected in all the samples.

Relative levels of *MT-I* mRNA were determined in total yolk sac RNA from normal heterozygous and homozygous *MTF-1* knockout embryos. Northern blotting revealed high levels of *MT-I* mRNA in the yolk sac RNA from normal embryos and diminished levels in that from heterozygous embryos. In contrast, little or none was detected in yolk sac RNA from homozygous embryos (Figure 3C). Actin mRNA levels were similar in these RNA samples.

The finding that E-box1 may play a role in regulating endoderm-specific expression of *MT-I* suggested that USF family members could be involved in this process (Li *et al.*, 1998). USF1 and USF2 have some overlapping biological activities (Sirito *et al.*, 1998) and both are expressed in the visceral endoderm (Figure 2B). The *in vivo* roles of these proteins were examined using *USF1* or *USF2* knockout mice (Figure 4). Homozygous *USF1* knockout mice are viable and fertile as adults (Sirito *et al.*, 1998). In contrast, ~50% of *USF2* null mutant mice die at birth (Sirito *et al.*, 1998). Nearly all *USF2* knockout males die as they become sexually mature and the few survivors are often infertile (M.Sawadogo and M.Sirito, unpublished data). Furthermore, embryos with targeted mutations of both the *USF1* and *USF2* genes die *in utero* (Sirito *et al.*, 1998). Homozygous null mutant *USF1* mice, or control mice with

Fig. 3. Homozygous knockout of *MTF-1* abolishes *MT* expression in the visceral endoderm. Heterozygous *MTF-1* knockout mice were inbred and embryos were harvested at day 12 of gestation. The *MTF-1* genotype of each embryo was determined by PCR. $+/+$, two normal *MTF-1* alleles; $+/-$, heterozygous for *MTF-1* knockout allele; $-/-$, homozygous for *MTF-1* knockout allele. The visceral yolk sacs and placentae were fixed and analyzed individually for MT, Sp1 or α -fetoprotein (α FP) using immunohistochemistry (A and B). D, deciduum basalis, ST, spongiotrophoblast layer. (A) Low- (100 $\times$) and high- (200 $\times$) magnification views of the immunohistochemical staining of MT in the visceral yolk sac. (C) Total RNA was extracted from the visceral yolk sac (four embryos) and analyzed by northern blot hybridization using *MT-I* and actin cRNA probes simultaneously on the same blot.

the same genetic background, were inbred and tissues were harvested at day 14 of pregnancy. Heterozygous *USF2* $^{+/-}$ mice were inbred and the littermates were individually genotyped at day 14 of pregnancy. Samples of identical genotype were pooled. Relative levels of *MT-I* mRNA were determined in total yolk sac RNA from control and homozygous *USF1* knockout embryos (Figure 4A) and from *USF2* (Figure 4B) knockout and normal embryo littermates. *MT-I* mRNA levels (relative to β -actin) were reduced 4.0-fold in yolk sacs from homozygous *USF1* knockout embryos relative to those from control embryos (Figure 4A), but were unaffected in the placenta (1.1-fold increase). In contrast, *MT-I* mRNA levels were essentially

Fig. 4. Homozygous knockout of *USF1*, but not *USF2*, attenuates *MT* expression in the visceral endoderm. Heterozygous *USF1* (A) or *USF2* (B) knockout and control embryos and tissues were harvested at day 14 of gestation. $+/+$, embryo with two normal *USF* alleles; $-/-$, embryo homozygous for *USF* knockout alleles. Total RNA was extracted from the visceral yolk sac (VYS) and placenta (six per genotype) and analyzed by northern blot hybridization using *MT-I* and actin cRNA probes simultaneously on the same blot. Hybrids were quantified by radioanalytical analysis of the membranes. These RNA samples were northern blotted at least three separate times with similar results.

unchanged (1.8-fold increase) in the yolk sacs from homozygous *USF2* knockout embryos (Figure 4B). Actin mRNA levels were similar in these yolk sacs. Although there was some degradation of this mRNA in the *USF2* knockout sample, this did not affect interpretation of the results (Figure 4B). The relative abundance of *MT-I* mRNA in the visceral yolk sac total RNA was identical among each of the control samples examined (*MTF* $^{+/+}$ littermates; *USF1* $^{+/+}$ genetic control and *USF2* $^{+/+}$ littermates). This suggests that *MT-I* expression in the visceral yolk sac is unaffected by the genetic background of the mouse strains in these experiments.

High-level *MT-I* expression in visceral endoderm is regulated by maternal dietary zinc

The effects of maternal dietary zinc deficiency during pregnancy on *MT-I* expression in the visceral endoderm were examined (Figure 5). Pregnant females were fed a normal diet or a zinc-deficient diet beginning on day 8 of pregnancy. RNA was extracted from visceral yolk sacs (~20 per sample) and placentae were collected at days 12 and 14 of pregnancy and analyzed by northern blotting (Figure 5A) with quantitative radio-analytic analysis of the membranes (Figure 5B). The results demonstrated that *MT-I* mRNA levels were reduced by ~40% on day 12 and by >20-fold on day 14 in mice fed the zinc-deficient diet (Figure 5). MT protein was reduced >10-fold, based on a [109 Cd]hemoglobin exchange assay (Andrews and Geiser, 1999) (data not shown). Zinc deficiency had little effect on placental *MT-I* mRNA levels (Figure 5).

Discussion

It has been known for >15 years that the mouse *MT-I* and *-II* genes are expressed at high levels only in a few, specific cell types in the developing conceptus (Andrews *et al.*, 1987a, 1993; Karasawa *et al.*, 1991; Liang *et al.*, 1996).

Fig. 5. Dietary zinc modulates *MT-I* expression in the visceral yolk sac. CD-1 mice were exposed to dietary zinc deficiency beginning on day 8 of pregnancy and the effects on *MT-I* mRNA levels in the visceral yolk sac and placenta were determined on days 12 and 14. Under these conditions, ~20% of the embryos developed abnormally by day 14, but there was no increase in embryo mortality (Andrews and Geiser, 1999). Total RNA was extracted from visceral yolk sacs (VYS) and placentae (~20 per sample) collected from mice fed a zinc-adequate (Zn-A) diet (+, containing 50 p.p.m. Zn^{2+}) or a zinc-deficient (Zn-D) diet (-, containing 1 p.p.m. Zn^{2+}). RNA was analyzed by northern blot hybridization using mouse *MT-I* and actin cRNA probes simultaneously. Hybrids were detected by autoradiography (A) and quantified by radioanalytic analysis of the membrane (B).

Although these genes are expressed at basal levels and are inducible by metal ions as early as the blastocyst stage of development (Andrews *et al.*, 1991), the mechanisms regulating cell-specific patterns of heightened expression during development remain undiscovered. The endoderm cells of the visceral yolk sac are the first cell type in the developing mouse embryo to activate the *MT-I* gene naturally and dramatically. This pattern of developmental activation of mouse *MT-I* gene expression is mimicked during the *in vitro* differentiation of F9 embryonal carcinoma cells along the visceral endoderm pathway (Andrews *et al.*, 1984). It was felt, therefore, that visceral endoderm expression of *MT* is a preprogrammed developmental event independent of maternal signals. In the present study we demonstrated that the transcription factor MTF-1, in cooperation with USF1, is essential for this cell-specific pattern of *MT-I* expression. MTF-1 is also essential for zinc and cadmium induction, as well as for basal expression of mouse *MT-I* in embryonic stem cells (Heuchel *et al.*, 1994) and in mouse embryo fibroblasts (Koizumi *et al.*, 1999), but these cell types express *MT-I* at high levels under normal culture conditions. Thus, it was unexpected that MTF-1 would play such a dominant role in regulating *MT-I* expression in the visceral endoderm. However, this finding, and the demonstration that dietary zinc status modulates endoderm expression of *MT-I*, strongly suggests that zinc is, in fact, the maternally derived factor responsible for this upregulation of *MT-I*. Zinc was also present in significant concentrations in the medium used to culture F9 cells.

The finding that maternal dietary zinc deficiency dramatically reduces the expression of *MT-I* in visceral endoderm cells is consistent with a prominent role of MTF-1 as a metalloregulatory sensor of zinc. The zinc-finger domain of MTF-1 has been highly conserved during evolution (Auf der Maur *et al.*, 1999), and the DNA-binding activity of native and recombinant MTF-1 is reversibly modulated by zinc interactions with the finger domain (Heuchel *et al.*, 1994; Dalton *et al.*, 1997). The zinc-fingers are heterogeneous in function and at least two exhibit low-affinity binding of zinc (Chen *et al.*, 1998, 1999). Dissection of the zinc-finger domain revealed that finger 1 of MTF-1 constitutes a unique metal-sensing domain that, in cooperation with the transactivation domains, produces a zinc-sensing metalloregulatory transcription factor (Bittel *et al.*, 2000).

The question remains as to why MTF-1 activity is zinc-activated naturally in visceral endoderm cells. It seems likely that the endoderm is particularly rich in zinc, perhaps due to an enhanced zinc-uptake system. The visceral yolk sac may play a role in the movement of zinc between the maternal and embryonic compartments. It has been shown to participate in the uptake of vitamins, amino acids and peptides (Padykula *et al.*, 1966). Although little is known about mechanisms of zinc uptake in mouse cells (Eng *et al.*, 1998), we recently demonstrated that the mouse *ZnT1* gene, which encodes a transmembrane protein that functions in the efflux of zinc (Palmiter and Findley, 1995), is highly expressed in the visceral yolk sac (Langmade *et al.*, 2000). *MTF-I* also modulates the level of expression of this gene in response to zinc (Langmade *et al.*, 2000). Thus, *MTF-I* may coordinate the expression of a cohort of genes involved in zinc homeostasis during development.

Another possible explanation for MTF-1 activity in the visceral endoderm is that, in these cells, there may be a unique set of co-activator proteins that facilitate activation of the *MT-I* gene. A novel finding in this study was that optimal expression of the *MT-I* promoter in the visceral endoderm required cooperative interactions between MTF-1 and USF1. Neither the proximal promoter (MREs) nor the distal promoter (E-box1) functioned alone, and mutations of the *USF1* gene significantly attenuated *MT-I* gene expression in visceral endoderm cells. Previous studies have implicated USF in the regulation of basal expression (Stuart *et al.*, 1984; Carthew *et al.*, 1987) and cadmium induction of the mouse *MT-I* gene (Li *et al.*, 1998). However, the E-box1 region of the *MT-I* promoter is constitutively occupied *in vivo* in several cell-types; *in vivo* footprinting studies and *in vitro* assays suggest that USF binds to this element (Li *et al.*, 1998). However, E-box1 has little effect on the basal expression or metal induction of the *MT-I* promoter in the few transfected cell types that have been studied (Li *et al.*, 1998). Our studies suggest that E-box1 may function in a cell-specific manner.

In spite of its ubiquitous expression in most cells, USF has been implicated in cell-specific regulation of the human insulin gene (Read *et al.*, 1993), the prostaglandin endoperoxide synthase-2 promoter in ovarian granulosa cells (Morris and Richards, 1996), pancreatic expression of a homeobox gene (*STF-1*) (Sharma *et al.*, 1996) and α -myosin heavy chain expression (Ojamaa *et al.*, 1995).

In each of these promoters, USF interacts with nearby elements to exert its effect, suggesting protein–protein interactions. Three isoforms of USF (USF1, USF2a and USF2b) have been described (Sirito *et al.*, 1992; Viollet *et al.*, 1996). The 43 kDa USF1 and the 44 kDa USF2 polypeptides are encoded by separate genes (Gregor *et al.*, 1990; Sirito *et al.*, 1994) while alternative splicing gives rise to the USF2a and USF2b isoforms (Viollet *et al.*, 1996). USFs (Sirito *et al.*, 1992; Viollet *et al.*, 1996) can interact with each other and with several different bZip transcription factors (Kurschner and Morgan, 1997; Pognonec *et al.*, 1997). Remarkably, USF1, but not USF2, was found to be important in regulating MT expression. This suggests that USF1 homodimers, or heterodimers not including USF2, are involved. Whether USF interacts directly with MTF-1 remains to be examined. The cell-specific nature of the cooperative interaction of E-box1 and MTF-1 suggests that protein(s) in addition to USF must also be important for optimal activation of *MT-I* expression in the visceral endoderm.

It is possible that chromatin structure and/or DNA methylation of the *MT-I* gene contribute significantly to its patterns of cell-specific expression. Inhibition of histone deacetylase activity renders cultured cells hypersensitive to induction of *MT* gene expression by metals (Andrews and Adamson, 1987), which suggests that the threshold for sensitivity to metals may have different set points in different cells based on nucleosome structure. In that regard, USF is also a component of the human β -globin locus control region (Bresnick and Felsenfeld, 1993) and may participate in the establishment of an active chromatin configuration (Salminen *et al.*, 1996). The mouse *MT-I* and *-II* genes are flanked by regions with clusters of DNase I hypersensitive sites that function as locus control regions, which permit copy number-dependent and integration site-independent expression of transgenes in transgenic mice (Palmiter *et al.*, 1993b). Our results demonstrate that these regions from the mouse *MT* locus are not essential for proper cell-specific expression of the *MT-I* promoter during early development. Interestingly, structural genes in the visceral endoderm are generally hypomethylated (Rossant *et al.*, 1986), and DNA methylation has been correlated with inactivation of the mouse *MT-I* gene (Lieberman *et al.*, 1983; Lu *et al.*, 1999). Thus, several mechanisms may contribute to regulating *MT* gene expression in the visceral endoderm.

A final interesting point of discussion is the finding that *MTF-1* is not essential for development and differentiation of the visceral yolk sac endoderm. Visceral endoderm cells lacking *MTF-1* had apparently normal morphology and normal expression of α -fetoprotein and actin. Targeted mutation of mouse *MTF-1* has revealed that it is an essential gene and homozygous null mutant embryos die on day 14 of development. The liver fails to develop properly in these mice, while development of the nervous system is not impaired (Günes *et al.*, 1998; Lichtlen *et al.*, 1999). Major functions of the fetal liver (e.g. serum protein synthesis and hematopoiesis) are performed earlier in development by the visceral yolk sac.

In summary, these studies demonstrated that MTF-1 is essential for the heightened expression of *MT-I* in the visceral endoderm of the developing mouse embryo and suggest that zinc modulates the ability of MTF-1 to

transactivate gene expression in these cells. It was also found that MTF-1 cooperates with USF1 to regulate endoderm-specific expression of the mouse *MT-I* gene.

Materials and methods

Animals

All experiments involving animals were conducted in accordance with NIH guidelines for the care and use of experimental animals and were approved by our Institutional Animal Care and Use Committee(s). The targeted disruption of *MTF-1* in the mouse has been described in detail previously (Günes *et al.*, 1998; Lichtlen *et al.*, 1999). Mice with targeted disruption of the mouse *USF1* or *USF2* genes have been described in detail previously (Sirito *et al.*, 1998). *MTF-1* or *USF2* heterozygous knockout mice were inbred. The day of a vaginal plug was considered day 1 of pregnancy. For each implantation site on day 14, the embryo, visceral yolk sac and placenta were harvested. For genotyping the conceptus, the DNA from each embryo was extracted and analyzed by PCR. Tissues of identical genotype were pooled for analysis. Mice homozygous for *USF1* knockout and a genetic control strain were generated by inbreeding littermates derived from inbred heterozygous *USF1* knockout mice. Offspring with two normal *USF1* alleles and those with two knockout *USF1* alleles were inbred; the visceral yolk sacs and placenta were harvested at day 14 of pregnancy.

For immunohistochemistry, the visceral yolk sac attached to the placenta was dissected out and fixed as described below. For RNA extraction, each visceral yolk sac was separated from the placenta, frozen in liquid nitrogen and stored at -80°C until genotype analysis was complete. Tissues of the same genotype (at least six yolk sacs each, from two females) were pooled and RNA was extracted as described below.

In experiments involving dietary zinc deficiency, mouse diets were purchased from Harlan Teklad (Madison, WI) and have been described in detail previously (Dalton *et al.*, 1996a; Andrews and Geiser, 1999). There was 0.5–1.5 $\mu\text{g/g}$ (1 p.p.m.) zinc in the zinc-deficient (Zn-D) diet and 50 $\mu\text{g/g}$ (50 p.p.m.) in the zinc-adequate (Zn-A) diet. These diets each contained $\sim 18 \mu\text{g/g}$ Cu^{2+} and were otherwise identical. CD-1 females (48–60 days old; Charles River Breeding Laboratories, Raleigh, NC) were mated with CD-1 males. On day 1 of pregnancy, mice were placed in pairs in cages and provided free access to Zn-A feed and deionized distilled water until day 8 of pregnancy. On day 8, pairs of mice were placed in cages with stainless steel false bottoms to reduce recycling of zinc (Cook Mills and Fraker, 1993) and the diet was changed to the Zn-D diet or mice were maintained on the Zn-A diet. On day 14, MT expression in placenta and visceral yolk sac was measured as described below. Embryonic tissues were pooled from ~ 20 implantation sites for each group. Samples were frozen in liquid nitrogen, stored at -80°C and later assayed in sextuplicate for ^{109}Cd -binding activity and *MT* mRNA levels.

In experiments involving *hPAP* transgenic mice, DNA microinjection and manipulation were carried out by the Transgenic and Gene Targeting Facility at the University of Kansas Medical Center. These transgenic experiments involved analysis of F_0 transgenic mice harvested on day 14 after embryo transfer. From each implantation site, the embryo was recovered for PCR genotyping of genomic DNA. The visceral yolk sac attached to the placenta was dissected out, the placenta was cross-sectioned and the tissues were frozen in liquid nitrogen and stored at -80°C until histochemical detection of *hPAP*, as described below. Routinely, 10–25% of these embryos were identified as transgenic.

Construction of *MT-I* promoter-*hPAP* fusions

The *MT-I* promoter constructs have been described in detail previously (Dalton *et al.*, 1994; Li *et al.*, 1998). Promoter constructs each provided the transcription start site and 66 bp of untranslated region. These were moved from CAT or luciferase reporter constructs (Dalton *et al.*, 1994; Li *et al.*, 1998) into the *hPAP* reporter vector described below.

To construct an *hPAP* reporter vector, a custom-made polylinker (*NotI*, *NheI*, *XhoI*, *HindIII*, *XbaI*, *NotI*) was first inserted between the *SacI* and *XbaI* sites of pGEM-7Zf(+) (Promega Corporation, Madison, WI), with elimination of the vector *XbaI* site. The 5.5 kb *HindIII*–*XbaI* fragment from pRSV-*hPAP* (American Type Culture Collection, Rockville Pike, MD) (Henthorn *et al.*, 1988; Knoll *et al.*, 1988; Lin and Culp, 1991), containing the complete *hPAP* gene (Henthorn *et al.*, 1988; Knoll *et al.*, 1988) and the bovine growth hormone 3' polyadenylation site, was then inserted between the corresponding sites in the polylinker. This provided the recipient for the following *MT-I* promoter constructs described previously: -742PAP , $-742\delta\text{PAP}$ (bases -43 to -150 inclusive are

deleted); -600PAP, -250PAP and -153PAP. These promoter fragments (through base +66) were excised with *NheI* (or *XhoI* depending on the parental plasmid) and *HindIII*, and inserted into these sites in the pGEM-7Zf-hPAP construct.

The E-box1:-153PAP fusion gene was constructed by inserting an E-box1 oligonucleotide duplex (exclusive of restriction-site bases: TGT-TCCACACGTCACATGGGTCGTCCTATCCGAGCC) between the *NheI* site and a *BglII* site adjacent to base -153 in -153PAP of the promoter insert. The E-box1 oligonucleotide encompassed bases -200 to -235 in the *MT-I* promoter (Li *et al.*, 1998).

To prepare DNA for microinjection, the ~6 kb *MT-I* promoter-hPAP-polyadenylation-site cassette was excised with *NotI* and separated from the 3 kb pGEM-7Zf vector by agarose gel electrophoresis. DNA was extracted from the gel using a Qiagen gel extraction kit and further purified by phenol-chloroform-isoamyl alcohol extractions. DNA was ethanol-precipitated and redissolved in microinjection buffer (10 mM Tris-HCl, 0.25 mM EDTA pH 7.4). DNA concentration was measured by fluorimetry.

Genotyping of embryos by PCR

PCR was used to determine the genotype of DNA from individual embryos using reaction conditions described previously (Lee *et al.*, 1996). The *MTF-1*, *USF1* and *USF2* knockout alleles were determined as described in detail previously (Heuchel *et al.*, 1994; Sirito *et al.*, 1998). Genotyping of F₀ *MT-hPAP* transgenic mice was performed using a sense-strand primer beginning at -5 bp in the mouse *MT-I* promoter (Searle *et al.*, 1984) and an antisense-strand primer beginning at +329 bp in hPAP (Knoll *et al.*, 1988).

Histochemical detection of hPAP

hPAP in whole mounts and tissue sections was detected as described (Deprimo *et al.*, 1996). Tissue samples were fixed in 2% (v/v) formaldehyde, 0.2% (v/v) glutaraldehyde in phosphate-buffered saline (PBS) at 4°C for 60 min. Samples were washed three times with cold PBS and then incubated in 1 ml of PBS for 45 min at 65°C to destroy endogenous peroxidase activity. Samples were then incubated for ≤18 h at 4°C in 1 ml of a freshly prepared solution of 1 mg/ml nitroblue tetrazolium, 1 mg/ml 5-bromo-1-chloro-3-indolyl phosphate (Promega) in 0.1 M Tris-HCl pH 10.0. For detection of hPAP in tissue sections, stained whole mounts were washed in PBS, dehydrated in ethanol, infiltrated and embedded in methacrylate using a JB-4 embedding kit (Polysciences; Worthington, PA). Sections (1–2 µm) were mounted on to poly-lysine-coated slides and not counterstained before photography.

Immunohistochemistry

A rabbit polyclonal antiserum against a synthetic peptide corresponding to the N-terminal 15 amino acids of mouse MT-I (N-Ac-MDPNASASTGGSATC-amide) was characterized previously (Dalton *et al.*, 1996b). Rabbit polyclonal antisera against Sp1 (PEP 2), USF1 and USF2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and antiserum against α -fetoprotein was purchased from Zymed Labs (San Francisco, CA).

Visceral yolk sacs and placentae were fixed overnight in Bouin's fluid at 4°C, embedded in paraffin, sectioned and used for light-level immunohistochemistry as described (Dalton *et al.*, 1996b). Slides were blocked for 1 h at room temperature with 10% goat serum in PBS-Triton X-100 and incubated overnight at 4°C with antisera against MT (1:50), Sp1 (1:100), α -fetoprotein (1:100), USF1 or USF2 (1:250). Slides were then incubated with anti-rabbit IgG conjugated to peroxidase and stained with a DAB kit (Zymed Labs, San Francisco, CA). Controls included non-immune serum and omission of primary antisera.

Extraction and northern blot analysis of RNA

Placentae and visceral yolk sacs (20 per group) from zinc-deficient or zinc-adequate mothers were rapidly thawed and RNA was extracted in buffers containing guanidinium isothiocyanate using a MIDI RNA isolation kit (Qiagen) according to the manufacturer's instructions. RNA from the visceral yolk sacs from knockout mice was isolated using a MINI RNA isolation kit according to the manufacturer's instructions (Qiagen). To remove residual DNA, all samples of total RNA were precipitated once with 3 M ammonium acetate at -2°C, followed by a final ethanol precipitation, as described (Andrews *et al.*, 1987b).

Total RNA (1 µg in 5 µl) was denatured and size separated by electrophoresis in a 0.75% agarose-formaldehyde gel. RNA was transferred and cross-linked to nylon membranes, and northern blots were prehybridized, hybridized and washed as described (Dalton *et al.*, 1994). Hybrids were detected by autoradiography at -70°C with

intensifying screens and quantified by radioimage analysis (Molecular Dynamics, Sunnyvale, CA). In each experiment, a duplicate gel was stained with acridine orange to verify integrity and equal loading of RNA, and blots were co-hybridized with *MT-I* and actin probes as an internal control.

The mouse *MT-I* cDNA used as a template for the synthesis of ³²P-labeled cRNA probe was described previously (Dalton *et al.*, 1994). The mouse actin cDNA template was purchased from Ambion (Austin, TX) and also used for the synthesis of a ³²P-labeled cRNA probe.

EMSA

EMSA was performed using nuclear extracts as described previously (Dalton *et al.*, 1996c, 1997; Bittel *et al.*, 1998; Li *et al.*, 1998). Proteins from nuclear extracts (5–10 µg in 2–3 µl) were incubated in buffer containing 12 mM HEPES pH 7.9, 60 mM KCl, 0.5 mM dithiothreitol, 12% glycerol, 5 mM MgCl₂, 0.2 µg poly(dI)/(dC)/µg protein and 8 fmol end-labeled double-stranded oligonucleotide (5000 c.p.m./fmol), in a total volume of 20 µl for 20 min on ice (Dalton *et al.*, 1996c, 1997). In antibody supershift experiments, 1 µg of USF1- or USF2-specific antibody was incubated with nuclear extracts for 2 h on ice before addition of the labeled binding site (data not shown). EMSA was performed using the E-box1 and mutant E-box1 oligonucleotides described in detail previously (Li *et al.*, 1998). An Sp1-binding site oligonucleotide and the MTF-1-binding site MREs have been described previously (Radtko *et al.*, 1993; Dalton *et al.*, 1996c). Protein-DNA complexes were separated electrophoretically and detected by autoradiography as described previously (Dalton *et al.*, 1997; Li *et al.*, 1998).

Measurement of MT protein levels

Steady-state levels of total MT were quantified in tissue homogenates using the [¹⁰⁹Cd]hemoglobin-exchange assay (Eaton and Cherien, 1991).

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