Structure, Function, and Regulation of a Subfamily of Mouse Zinc Transporter Genes*

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Zinc is an essential metal for all eukaryotes, and cells have evolved a complex system of proteins to maintain the precise balance of zinc uptake, intracellular storage, and efflux. In mammals, zinc uptake appears to be mediated by members of the Zrt/Irt-like protein (ZIP) superfamily of metal ion transporters. Herein, we have studied a subfamily of zip genes (zip1, zip2, and zip3) that is conserved in mice and humans. These eight transmembrane domain proteins contain a conserved 12-amino acid signature sequence within the fourth transmembrane domain. All three of these mouse ZIP proteins function to specifically increase the uptake of zinc in transfected cultured cells, similar to the previously demonstrated functions of human ZIP1 and ZIP2 (Gaither, L. A., and Eide, D. J. (2000) J. Biol. Chem. 275, 5560–5564; Gaither, L. A., and Eide, D. J. (2001) J. Biol. Chem. 276, 22258–22264). No ZIP3 orthologs have been previously studied. Furthermore, this first systematic comparative study of the in vivo expression and dietary zinc regulation of this subfamily of zip genes revealed that 1) zip1 mRNA is abundant in many mouse tissues, whereas zip2 and zip3 mRNAs are very rare or moderately rare, respectively, and tissue-restricted in their accumulation; and 2) unlike mouse metallothionein 1 and zip4 mRNAs (Dufner-Beattie, J., Wang, F., Kuo, Y.-M., Gitschier, J., Eide, D., and Andrews, G. K. (2003) J. Biol. Chem. 278, 33474–33481), the abundance of zip1, zip2, and zip3 mRNAs is not regulated by dietary zinc in the intestine and visceral endoderm, tissues involved in nutrient absorption. These studies suggest that all three of these ZIP proteins may play cell-specific roles in zinc homeostasis rather than primary roles in the acquisition of dietary zinc.

Zinc is an essential trace element that is required for the catalytic activity of numerous metalloenzymes (4, 5) and can also serve a purely structural role by stabilizing the conformation of certain zinc-dependent protein domains, such as zinc fingers, zinc clusters, and RING fingers, that are commonly found in transcriptional regulatory proteins (5, 6). Deficiency of this essential metal can have devastating effects. In mammals, inadequate levels of zinc in the diet lead to dermatologic lesions, growth retardation, mental disorders, and compromised function of the immune and reproductive systems (7–9). Likewise, high levels of zinc can be cytotoxic. Thus, cells must maintain tight control over intracellular zinc levels. This control is achieved through a balance of zinc efflux, cellular zinc storage, and zinc uptake. Each of these activities is mediated by a distinct family of proteins (8).

In mammals, the zinc transporter (Znt) family of proteins function in a tissue-, cell-, and organelle-specific manner to regulate intracellular zinc homeostasis. These proteins contain six predicted transmembrane domains and are thought to function as multimers. Seven members of the Znt family have been identified to date in mammals (Znt1–7), and genetic studies have confirmed the importance of many of these genes in mammalian zinc metabolism (10–17). Recent studies demonstrated that zinc can regulate expression of the Znt1 gene (18), and dietary zinc modulates Znt1 and Znt2 mRNA levels in the kidney, intestine, and liver (19). Zinc can also regulate the intracellular localization of the ZnT4 and ZnT6 proteins (18).

Intracellular zinc is bound by small cysteine-rich proteins called metallothioneins (MTs) (20). In the mouse, the MT family consists of four members. Exposure to high levels of zinc causes MT accumulation through increased gene expression, whereas dietary zinc deficiency leads to decreased MT abundance through decreased gene expression and protein destabilization (21). These proteins are thought to sequester zinc when present at high levels, protecting against heavy metal toxicities, and to provide a labile pool of zinc under limiting conditions that can be released for use by other proteins.

In eukaryotes, uptake of several essential metals is mediated by members of the Zrt/Irt-like protein (ZIP) superfamily of metal ion transporters (22, 23). The first member of this family to be identified was iron-regulated transporter-1 (Irt1) from Arabidopsis. Irt1 was found to preferentially import iron, but can also transport manganese, cadmium, and zinc (24–27). Subsequently, two other members of this superfamily (zinc-regulated transporter-1 (Zrt1) and Zrt2) were identified that function in zinc uptake in yeast (28, 29). The acronym for the superfamily (i.e. ZIP) reflects Zrt- and Irt-like proteins. ZIP proteins contain eight putative transmembrane domains with conserved histidine, serine, and glycine residues within the fourth transmembrane domain, which may play a role in metal binding during transport. These proteins also contain a

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† The abbreviations used are: ZnT, zinc transporter; MT, metallothionein; ZIP, Zrt/Irt-like protein; Irt, iron-regulated transporter; Zrt, zinc-regulated transporter; EST, expressed sequence tag; RT, reverse transcription; HA, hemagglutinin; BAC, bacterial artificial chromosome.
characterization of a subfamily of zinc transporters

Although several recent studies have examined the expression and regulation of members of this conserved subfamily of zinc transporters, there have been no systematic comparative studies of their expression or regulation in vivo. The expression of all members of this subfamily of three zinc transporters, ZIP1 (SLC39A1), ZIP2 (SLC39A2), and ZIP3 (SLC39A4) is regulated by transcriptional and post-transcriptional mechanisms. ZIP1 mRNA has been detected in adult mouse testes (32), and ZIP3 mRNA is also found in most human tissues (2) and cultured human cell lines. In contrast, ZIP2 mRNA was not detectable by Northern blot analysis of human tissue RNAs (1). Expression of ZIP3 has not been examined in any system, and expression of ZIP1 and ZIP2 has not been examined in adult mouse tissues. A modest hormonal regulation of ZIP1 mRNA in cultured prostate cell lines has been reported (33); and in human monocytes and TPH-1 cells treated with a zinc chelator, ZIP2 mRNA abundance increased significantly, whereas ZIP1, ZIP3, and ZIP4 mRNA abundance did not change (34, 35). The objective of the studies presented herein was to characterize in detail the structure and function of the mouse ZIP1, ZIP2, or ZIP3 genes and to compare and contrast their regulation in vivo in nutrient-absorptive tissues (intestine and embryonic visceral endoderm) during periods of dietary zinc deficiency.

EXPERIMENTAL PROCEDURES

Computer Analysis of Sequence Data—Multiple sequence alignments were performed using the Aligned program within the Vector NTI Suite program (Informax, Bethesda, MD).

Plasmid Construction—The expressed sequence tags (ESTs) for ZIP1 (GenBank accession number BE572590) and ZIP3 (accession number BF100710) were obtained from Incyte Genomics (St. Louis, MO). Sequencing of these ESTs revealed that they contain the expected open reading frames. The mouse ZIP2 cDNA was obtained by reverse transcription (RT)-PCR from poly(A)-enriched RNA isolated from the placenta of day 12 zinc-deficient pregnant mice (described below). Reverse transcription was carried out using Improm-II reverse transcriptase (Promega, Madison, WI), and PCR of the open reading frames was carried out using Flu DNA Polymerase (Stratagene, La Jolla, CA). For zinc uptake studies, selectable mammalian pCDNA3.1-Puro (+) expression plasmids (36) encoding each of the three mouse ZIP proteins with C-terminal hemagglutinin (HA) tags were constructed through a multistep cloning strategy. The resulting plasmids contained the open reading frames for uptake experiments were washed three times with ice-cold distilled water. On day 8, mice were placed in pairs in cages with stainless steel false bottoms to reduce the recycling of zinc (42). Water bottles were washed with 4 x HCl and rinsed with deionized water to remove zinc. The diet was changed to the zinc-deficient diet, or where indicated, the zinc-aciduate diet was maintained. The visceral yolk sacs, placenta, and maternal small intestines were harvested on days 11–15 (six mice per group) and snap-frozen in liquid nitrogen for RNA extraction and subsequent Northern analysis or RT-PCR.

RNA Extraction and Northern Analysis—Total RNA was isolated either using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions or as described previously (18, 40). Polyadenylated RNA was prepared from total RNA using the Oligotex mRNA isolation kit (QIAGEN Inc., Valencia, CA) according to the manufacturer’s instructions. Total RNA (3 µg/lane) was fractionated on formaldehyde-containing 1% agarose gels, transferred to nylon membranes, and immobilized via UV cross-linking (43). Duplicate gels were stained with acridine orange to monitor RNA integrity and to normalize for loading.

DNA fragments containing full-length zip1, zip2, or zip3 cDNA were excised from plasmids and used as probes for Northern blot hybridization. Probes were labeled with [32P]dCTP using the Random Primers DNA labeling system (Invitrogen) according to the manufacturer’s instructions. The mouse MT-1 probe was as described previously (38). Membranes were hybridized and washed under stringent conditions as described previously (18, 38). Hybrids were detected by autoradiography with intensifying screens at –70 °C.

Cell Culture and Transient Transfections—HEK293 cells were maintained in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine at 37 °C in a humidified 5% CO2 incubator. For transient transfections, cells were seeded onto poly-l-lysine-treated 24-well plates at a density of 2 × 105 cells/well and were transfected the following day. Transfections were carried out using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Zinc uptake assays were performed 36–48 h later.

Zinc Uptake Assays—Zinc uptake assays were performed as described previously (1, 2). After transfection, cells were washed twice with cold uptake buffer (15 mM Hepes, 100 mM glucose, and 150 mM KCl, pH 7.0), followed by a 10-min incubation with prewarmed uptake buffer. Cells were then incubated in prewarmed uptake buffer containing the specified concentration of 65ZnCl2 (PerkinElmer Life Sciences) in a 37 °C shaking incubator for 15 min unless otherwise indicated. Assays were stopped by addition of an equal volume of cold uptake buffer supplemented with 1 mM EDTA (stop buffer). Cells were collected by filtration on glass fiber filters (Type A/E, Gelman Sciences) and washed three times with stop buffer (total of 10 ml). Cell-associated radioactivity was measured with a Packard Auto-Gamma 5650 γ-counter. Metal salts were obtained from Sigma. Stock solutions of the chelators of various metals (CoCl2, NiCl2, CuCl2, CdCl2, and NiCl2) and AgNO3 were prepared at 100 mM in distilled water. A ZnCl2 stock was prepared at 100 mM in 0.2 N HCl, and a FeCl3 stock was prepared at 50 mM in 0.1 HCl. Sodium ascorbate (1 mM) was used to reduce Fe3+ to Fe2+. Ascorbate treatment alone did not alter zinc uptake activity (data not shown). Cells grown in parallel to those used for the assays were washed three times with uptake buffer, resuspended in phosphate-buffered saline containing 0.1% SDS and 1% Triton X-100 for cell lysis, and then assayed for protein content using a Bradford assay kit. Zinc accumulation and uptake rates were normalized to protein concentrations of these cell lysates. Michaelis-Menten constants were determined by nonlinear interpolation of the data using Prism (Version 3.0a for Macintosh, GraphPAD Software, San Diego, CA).

RT-PCR Detection of Mouse zip2 mRNA—Tissue-specific expression of zip2 was examined by RT-PCR. Total RNA from various mouse tissues (1 µg/reaction) was DNase I-treated according to the manufac-
transcriptase. Samples were then amplified using Platinum Taq DNA polymerase (Stratagene) for 30 cycles. Mouse ESTs corresponding to this shorter transcript suggests that non-canonical polyadenylation signal (Fig. 2). The number of ESTs corresponding to this shorter transcript suggests that this message is significantly less abundant than the longer message. As this alternative poly(A) tail occurs within the 3’- untranslated region, the same protein is predicted to be encoded by both messages. Both zip1 and zip3 cDNAs contain a thymidine-rich region within their long 3’-untranslated regions, but the significance of this is not yet known.

Comparison of the zip1 cDNA and gene sequences initially revealed a zip1 pseudogene within this BAC in the data base. The pseudogene has no introns, terminates in a poly(A) tract, and displays mismatches and codon deletions compared with the EST sequences. To identify a BAC containing the actual expressed zip1 gene, primers were designed to differentiate between the functional gene and the pseudogene based on the sequence mismatches. These primers were sent to Incyte Genomics for PCR screening of a mouse genomic BAC library. Two positive clones were identified, and partial sequence analysis revealed that they were devoid of the nucleotide mismatches seen in the pseudogene. These BACs were then sequenced in their entirety by the Mouse Genomic Sequencing Group of the University of Oklahoma.

The exon-intron structure for each gene was determined by aligning the gene with the cDNA and by identifying splice donor and acceptor consensus sequences (Fig. 2). All three genes are relatively small, spanning <10 kilobase pairs of DNA, and contain four exons. Using accession numbers for the BACs containing each zip gene, the chromosomal localization was assigned using the mouse genome server. Localization of zip1 to chromosome 3 is consistent with a previous report (32). The zip2 gene was localized to chromosome 14, the zip3 gene to chromosome 10, and the zip1 pseudogene to chromosome 2.

Analysis of the cDNA sequence revealed that the predicted mouse ZIP1, ZIP2, and ZIP3 proteins are 324, 309, and 317 amino acids in length, respectively. Alignment of the predicted human and mouse ZIP proteins revealed significant sequence conservation along the entire length of each of the three proteins (Fig. 3). The mouse and human ZIP1, ZIP2, and ZIP3 proteins are 98, 78, and 83% identical, and 96, 85, and 90% similar, respectively. Computer analysis of the zip3 cDNA identified an open reading frame for mouse ZIP3 that contains an additional 31 amino acids N-terminal to the initiator methionine shown in Fig. 3C. This extension is due to the presence of an in-frame methionine codon located 93 nucleotides further from the translational initiation codon.
upstream. This potential upstream start codon does not adhere to the Kozak consensus sequence for optimal translation (37), whereas the downstream codon does. In addition, this N-terminal extension is not present in the human ortholog. We therefore conclude that the downstream methionine is the initiator codon that is used in vivo, although this remains to be proven.

Putative transmembrane domains for the mouse ZIP proteins were predicted using the on-line application TMPred. Consistent with other members of the ZIP superfamily of metal ion transporters, these three mouse ZIP proteins contain eight putative transmembrane domains (Fig. 3).

Mouse zip1, zip2, and zip3 Each Encode Functional Zinc Transporters—Human ZIP1 and ZIP2 have been shown to

3 Available at www.isrec.isb-sib.ch.
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function in zinc uptake, but the function of ZIP3 has not been reported. To examine the functions of mouse ZIP1, ZIP2, and ZIP3, the open reading frame for each of these ZIP proteins was cloned into the mammalian expression vector pcDNA3.1Puro, and a linker encoding an HA tag was cloned in-frame immediately preceding the stop codon. HEK293 cells were transiently transfected with either empty vector or a vector encoding one of the three HA-tagged mouse ZIP proteins. Pools of transfectants were assayed for $^{65}$Zn uptake activity in the presence of either a 10- or 50-fold molar excess of competitor metal (Fig. 5). Zinc uptake by the endogenous system in HEK293 cells was strongly inhibited by excess zinc, copper, and cadmium and to a lesser extent by other metals (Fig. 5A). Consistent with the hypothesis that these three mouse ZIP proteins function as zinc transporters, zinc uptake by mouse ZIP1, ZIP2, and ZIP3 was strongly inhibited by the addition of excess zinc (Fig. 5, B–D). Zinc uptake by mouse ZIP1 and ZIP2 was only modestly inhibited by other metals. Conversely, zinc uptake by mouse ZIP3 was inhibited by all metals tested, except for iron, suggesting that it may be more promiscuous in its substrate selection. Nonetheless, all three appear to be largely zinc-specific.

Expression Patterns of Mouse zip1, zip2, and zip3 Are Unique, but Are Not Responsive to Dietary Zinc in the Adult Intestine and Embryonic Visceral Endoderm—To examine the tissue-specific patterns of expression of zip1, zip2, and zip3, Northern blot analysis was carried out using total RNA extracted from several organs from male and female CD-1 mice as well as from extraembryonic tissues surrounding the developing mouse embryo. Expression of zip1 was detected in all tissues examined, except for the pancreas, with the highest levels of expression seen in the intestine and ovary (Fig. 6A, upper panel). The integrity of the pancreatic RNA was confirmed by staining a duplicate gel with acridine orange (Fig. 6A, lower panel). In contrast to zip1, expression of zip3 was tissue-restricted, with the highest level seen in the testes (Fig. 6A, middle panel). In addition, the abundance of zip3 mRNA was much lower than that of zip1 mRNA in all tissues, except in the testes.

Despite repeated attempts, including the use of purified polyadenylated RNA, zip2 was not detected by Northern blotting. Therefore, the more sensitive method of RT-PCR was used to identify tissues that express the zip2 gene (Fig. 6B, upper panel). Similar to zip3, expression of zip2 appeared to be tissue-restricted, with the highest levels detected in the skin, liver, ovary, and visceral yolk sac. As a control for reverse transcription, zip1 was amplified from the same reactions (Fig. 6B, lower panel). These data support the results from the Northern blot experiments shown in Fig. 6A, demonstrating the nearly ubiqui-

mouse ZIP1, ZIP2, and ZIP3 was virtually identical over the time course of this experiment. Only low levels of zinc accumulation were detected when these assays were carried out at 0 °C, indicating that zinc accumulation by each of these three mouse ZIP proteins as well as by the endogenous system is temperature-dependent and therefore is likely to be transport-er-mediated rather than due to zinc binding to the cell surface. Consistent with this, uptake by the endogenous system as well as by mouse ZIP1, ZIP2, and ZIP3 was concentration-dependent and saturable. When assayed over a range of zinc concentrations, the uptake activities of mouse ZIP1, ZIP2, and ZIP3 showed surprisingly similar Michaelis-Menten kinetics, with apparent $K_m$ values of 1.7, 1.6, and 1.6 μM, respectively, and $V_{max}$ values of 9.5–10.5 pmol of zinc/min/mg of protein. Compared with mouse ZIP1, ZIP2, and ZIP3, the endogenous system in HEK293 cells had a similar apparent $K_m$ of 2.0 μM, but a far lower $V_{max}$ of 4.1 pmol of zinc/min/mg of protein, confirming that its activity is distinguishable from that of mouse ZIP1, ZIP2, and ZIP3.

To assess the substrate specificity of uptake by mouse ZIP1, ZIP2, and ZIP3, we examined the effect of various metals on zinc accumulation by cells expressing each of the three mouse ZIP proteins. HEK293 cells were transiently transfected with either empty vector or a vector encoding one of the three HA-tagged mouse ZIP proteins. Pools of transfectants were assayed for $^{65}$Zn uptake activity in the presence of either a 10- or 50-fold molar excess of competitor metal (Fig. 5). Zinc uptake by the endogenous system in HEK293 cells was strongly inhibited by excess zinc, copper, and cadmium and to a lesser extent by other metals (Fig. 5A). Consistent with the hypothesis that these three mouse ZIP proteins function as zinc transporters, zinc uptake by mouse ZIP1, ZIP2, and ZIP3 was strongly inhibited by the addition of excess zinc (Fig. 5, B–D). Zinc uptake by mouse ZIP1 and ZIP2 was only modestly inhibited by other metals. Conversely, zinc uptake by mouse ZIP3 was inhibited by all metals tested, except for iron, suggesting that it may be more promiscuous in its substrate selection. Nonetheless, all three appear to be largely zinc-specific.

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Fig. 4. Characterization of zinc uptake kinetics in transfected cells expressing mouse ZIP1, ZIP2, or ZIP3. HEK293 cells were transiently transfected with either empty expression vector (pcDNA3.1Puro (+)) or the expression vector for HA-tagged mouse (m) ZIP1, ZIP2, or ZIP3 (pcDNA3.1-zip1-HA, pcDNA3.1-zip2-HA, or pcDNA3.1-zip3-HA, respectively). Transfectants were cultured for 48 h prior to assay. A, zinc accumulation was assayed in cells expressing vector only (○ and △), ZIP1-HA (● and ▽), ZIP2-HA (▼ and ▼), or ZIP3-HA (■ and □) in the presence of 2 μM $^{65}$Zn at 37 °C (closed symbols) or 0 °C (open symbols) for the indicated times. B, the concentration dependence of zinc uptake activity was determined over a range of zinc concentrations in transfected cells incubated with $^{65}$Zn at 37 °C for 15 min. Each point represents the mean of a representative experiment (n = 3), and error bars indicate ±S.D.
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Our previous studies also demonstrated that expression of **zip1** mRNA was reduced in response to dietary zinc deficiency (Fig. 7A, third panel). **zip1** mRNA abundance did not change in response to dietary zinc deficiency in either the maternal small intestine or the embryonic visceral yolk sac (Fig. 7A, first panel). Similarly, **zip3** mRNA levels did not change in either of these tissues under these conditions (Fig. 7A, second panel). The two transcripts seen for **zip3** in Fig. 7 presumably result from the presence of the two different polyadenylation signals, and the relatively low intensity of the smaller band correlates with the fewer number of ESTs with the alternative poly(A) tail that results in a significantly shorter transcript.

Semiquantitative RT-PCR was used to examine **zip2**, **MT-I**, and **zip1** mRNA abundance during dietary zinc deficiency. Total RNA was reverse-transcribed and subsequently PCR-amplified for increasing numbers of cycles. As shown in Fig. 7B, when the PCR products were first detectable by agarose gel electrophoresis (21 cycles), the abundance of the product directly reflected the relative abundance of **MT-I** mRNA as determined by Northern blotting (Fig. 7A, third panel). As expected, these differences in abundance diminished with increasing cycle number. This experiment was then adapted to analyze **zip2** mRNA abundance in response to zinc deficiency using **zip1** as a control for reverse transcription and as a control for an mRNA whose abundance does not change under these conditions (Fig. 7A, first panel). PCR products for **zip1** were detectable after 27 amplification cycles, and their abundance did not change during dietary zinc deficiency, consistent with the Northern blot results (Fig. 7, compare C with A). Amplification products for **zip2** were detectable after 30 cycles (Fig. 7C), and their abundance also apparently did not change during dietary zinc deficiency. Modest changes (−2-fold) in **zip2** mRNA abundance may not have been detected under these experimental conditions.

**DISCUSSION**

The studies reported herein focused on a subfamily of three mouse **zip** genes, two of whose human orthologs have been demonstrated to function as zinc transporters (1, 2). As in humans, there appears to be only three members of this subfamily in the mouse. Given the high degree of amino acid

divalent cations. The **hatched bars** represent 65Zn uptake in the absence of competitor. Each point represents the mean of a representative experiment (n = 3), and **error bars** indicate ±S.D. m, mouse.
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similarity to their human orthologs, we predicted mouse ZIP1 and ZIP2 to have very similar functional characteristics, as was found to be the case. However, we did note several differences. First, human ZIP1 and ZIP2 function as zinc transporters, but their uptake is strongly inhibited by other metals (1, 2). Zinc uptake by human ZIP1 is inhibited by copper, iron, and cadmium, and that by human ZIP2 is inhibited by all other metals tested. In contrast, uptake by mouse ZIP1 and ZIP2 reported here was relatively specific for zinc as a substrate, with only modest inhibition by several other metals. These differences in uptake properties could reflect, in part, differences inherent in the cell types that were assayed. The data presented herein for mouse ZIP3 zinc uptake properties and metal specificity reported here is the case in human tissues (1), ZIP1 is expressed in nearly all tissues (e.g., liver, skin). However, higher transcript levels were detected in the ovary, liver, and skin. In contrast, ZIP3 mRNA was generally more abundant in these tissues compared with ZIP2 mRNA, but it was also present at the highest levels in only a few tissues (e.g., testes). However, those tissues that expressed ZIP2 and/or ZIP3 also expressed ZIP1. Interestingly, all three of these transcripts could be detected in the adult intestine and embryonic visceral yolk sac. This could represent a redundancy that is necessary in case one of the ZIP genes is inactivated by mutation. Alternatively, it is possible that they

Fig. 6. Tissue-specific expression of the mouse zip1, zip2, and zip3 genes. Total RNA was isolated from the indicated mouse (m) tissues. A, RNA was resolved by formaldehyde-agarose gel electrophoresis (3 μg/lane), transferred to nylon membranes, and probed with cDNA probes against either zip1 (upper panel) or zip3 (middle panel). A duplicate gel was stained with acridine orange (lower panel) to verify RNA integrity and to normalize loading. B, RNA was reverse-transcribed (1 μg/reaction) and subsequently PCR-amplified using primers corresponding to zip2 (upper panel) or zip1 (lower panel). Plasmids containing the appropriate cDNAs were used as positive controls, and no input template was used as a negative control. Amplification of zip2 yielded a 406-bp product, whereas amplification of zip1 yielded a 1000-bp product. sm int, small intestine.

Fig. 7. Effects of dietary zinc deficiency on mouse zip1, zip2, and zip3 mRNA abundance in the maternal small intestine and visceral yolk sac. Pregnant CD-1 mice were fed either a zinc-adequate (+) or zinc-deficient (−) diet on day 8 of pregnancy. Maternal small intestines and visceral yolk sacs were collected on days (d) 11–15 of pregnancy (i.e., days 3–7 of the zinc-deficient diet). A, RNA was isolated; resolved by formaldehyde-agarose gel electrophoresis (3 μg/lane); transferred to a nylon membrane; and hybridized with labeled cDNA probes for zip1, zip3, and MT-I. B, RNA was isolated, reverse-transcribed (1 μg/reaction), and subsequently PCR-amplified using primers corresponding to MT-I mRNA to produce a 138-bp product. Amplification was carried out for the indicated number of cycles. No input template (NT) was used as a negative control. C, RNA from the embryonic visceral yolk sac was reverse-transcribed (1 μg/reaction) and subsequently PCR-amplified using primers corresponding to zip2 or zip1 to control for efficiency of reverse transcription. Amplification was carried out for 27, 30, or 33 cycles. Plasmids containing the appropriate cDNAs were used as positive controls (PT), whereas no input template (NT) was used as a negative control. Amplification of zip2 yielded a 406-bp product, whereas amplification of zip1 yielded a 1000-bp product. m, mouse.
are expressed in the same tissue, but within different subsets of cells within that tissue. This could be addressed through immunohistochemistry using antibodies specific for each ZIP protein.

Another possibility to explain the large number of zip genes in the mouse genome is that different ZIP proteins may exhibit distinct subcellular localization that represents functional differences. This is seen in the case of yeast ZIP proteins. Zrt1 and Zrt2 function at the plasma membrane to import zinc into cells, whereas Zrt3 functions to transport stored zinc out of the yeast vacuole during the transition from zinc-replete to zinc-limiting conditions (28, 29, 44). A similar scenario is seen in the ENaT family of zinc transporters. ENaT1 functions to export zinc from cells and is localized to the plasma membrane (10), whereas ZnT2, ZnT3, ZnT6, and ZnT7 are located within intracellular membranes, causing zinc accumulation within vesicles or organelles (11, 16, 45–47).

Although many ZIP proteins are known to transport zinc, yet another possibility for the existence of multiple zip genes is that they may differ in the metals that they transport within the physiological context of the animal. There is precedence for this in the case of the Arabidopsis ZIP protein Irt1, whose preferential substrate is iron, but it can also transport manganese, cadmium, and zinc (24, 26, 27). As mentioned above, the mouse ZIP1, ZIP2, and ZIP3 transporters were found to be largely zinc-specific.

Contrary to what we expected, zip1, zip2, and zip3 mRNA abundance was not regulated in vivo by dietary zinc in tissues that function in the absorption of dietary or maternal zinc. Expression of genes encoding other zinc transporters, such as members of the zip and Znt families, is regulated, at least in part, at the RNA level by their substrate. The first zip gene to be identified, which encodes the Arabidopsis Irt1 iron transporter, is induced in roots under conditions of iron deficiency (23). Likewise, expression of zip genes encoding the yeast Zrt1 and Zrt2 and Arabidopsis ZIP1, ZIP3, and ZIP4 zinc transporters is induced under conditions of zinc deficiency (28, 29, 48). In addition, mouse zip4, the ortholog of human ZIP4, which is mutated in the genetic human zinc metabolism disorder acrodermatitis enteropathica, is zinc-regulated in the mouse intestine and visceral yolk sac (3). Zinc deficiency causes a marked increase in expression of zip4 in the intestine, which is consistent with the finding of increased intestinal zinc uptake activity during periods of dietary zinc deficiency in mammals (19, 49–52). However, zip1 mRNA is also relatively abundant in the mouse intestine and may thus be responsible, at least in part, for the constitutive zinc uptake activity found in the zinc-replete intestine. Alternatively, zip1 may be regulated by other signals. For example, human ZIP1 may be hormonally regulated in cultured prostate cells (33).

The lack of zinc responsiveness of the mouse zip2 and zip3 genes and the low abundance of zip2 and zip3 mRNAs in tissues that function in the absorption of dietary zinc are consistent with the concept that these proteins may serve cell-specific roles in zinc homeostasis. A zinc chelator has been shown to increase the abundance of ZIP2 mRNA in cultured monocytes and THP-1 cells (35). Thus, it is possible that zip2 and zip3 may be regulated by zinc in a cell-specific manner. Whether dietary zinc can modulate the cell-specific expression of the zip2 and zip3 genes in vivo in tissues other than the intestine and visceral yolk sac remains to be determined.

The lack of regulation of mouse zip1, zip2, and zip3 mRNA abundance by zinc certainly does not preclude the possibility that they are functionally zinc-responsive. It is possible that the mouse ZIP1, ZIP2, and ZIP3 proteins are regulated by translational and/or post-translational mechanisms in response to zinc. The intracellular trafficking of the human Menkes’ copper transporter is regulated by copper (53). Under conditions of low copper, the protein is localized in the trans-Golgi network; but when copper levels are elevated, the protein rapidly relocates to the plasma membrane (54–56). Whether the intracellular trafficking of these mouse ZIP proteins is zinc-responsive is under investigation. It is also possible that the uptake activity of these ZIP proteins may be regulated, possibly through post-translational modifications such as phosphorylation, circumventing the need for regulation at the RNA level. The structural basis for the zinc uptake activity of ZIP proteins is not understood.

The data presented herein describe a subfamily of mouse zip genes that can function as zinc transporters and whose expression is differentially regulated in a tissue-specific manner. These results suggest that these genes may function in mammalian zinc homeostasis, and genetic manipulation of these genes in the mouse model system will allow us to directly test this hypothesis.

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