Splice Variants of the Human Zinc Transporter ZnT5 (SLC30A5) Are Differentially Localized and Regulated by Zinc through Transcription and mRNA Stability*

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Kelly A. Jackson‡, Rachel M. Helston‡, Jill A. McKay‡, Elaine D. O’Neil‡, John C. Mathers‡, and Dianne Ford†‡

From the Human Nutrition Research Centre, ‡Institute for Cell and Molecular Biosciences, and †School of Clinical Medical Sciences, Newcastle University, Newcastle upon Tyne NE2 4NN, United Kingdom

Maintenance of cellular zinc homeostasis includes regulating the expression of cell membrane zinc transporters. Knowledge about the mechanisms underlying changes in mammalian zinc transporter mRNA abundance is poor. We demonstrated that when expressed in Chinese hamster ovary cells as N-terminal fusions to green fluorescent protein, two splice variants of ZnT5 adopt different subcellular locations (either in the Golgi apparatus or throughout the cell, including at the plasma membrane) indicating discrete roles in cellular zinc homeostasis. We demonstrated, using a β-galactosidase reporter gene, that both splice variants were expressed from a promoter region that was transcriptionally repressed by increased extracellular zinc (150 μM compared with 3 μM; ~40%) and by extracellular zinc depletion, using the chelator N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (~20%). We mapped the zinc-responsive element to the region ~154 to +50, relative to the predicted start of transcription, and showed that a consensus metal response element sequence (~410 to ~404) was not responsible for these effects. Changes in ZnT5 mRNA abundance in Caco-2 cells at different zinc concentrations were in parallel to the changes in promoter activity (~40% reduction at 150 μM zinc) but in the presence of actinomycin D, to prevent transcription, we observed a marked stabilization (1.7–2-fold accumulation over 24 h) of ZnT5 mRNA. We conclude that effects of zinc on ZnT5 transcription and mRNA stability act in opposition to balance mRNA abundance for cellular zinc homeostasis. To our knowledge, this is the first report that zinc affects the stability of a transcript with a direct role in cellular zinc homeostasis.

It is estimated that between 3 and 10% of human genes code for proteins with zinc binding domains (1, 2). These proteins include those in which zinc plays a role in protein structure, typified by the zinc finger domains of nucleic acid-binding proteins such as transcription factors, and those in which the redox stability and ability of zinc to form polyhedral coordination complexes with a variety of ligands is exploited in catalysis. Whole body and cellular zinc homeostasis is, therefore, essential and is achieved through mechanisms that include regulating the expression of cell membrane zinc transporters. Human zinc transporters are classified in two unrelated families, SLC30 and SLC39 (3, 4). The prevailing view is that members of the SLC30 family transport zinc in directions, which reduce cytosolic concentration, specifically out of the cell across the plasma membrane or into intracellular compartments, and that members of the SLC39 family function in the opposite direction to increase cytosolic zinc concentration by uptake across the plasma membrane or efflux form intracellular compartments.

ZnT5 (SLC30A5) was cloned simultaneously by two groups, including our own (5, 6). We reported that ZnT5 is expressed at the apical membrane of the human intestinal Caco-2 cell line, initially determined by expression of the Myc-tagged protein from an expression construct (5) but confirmed subsequently for the native protein both in Caco-2 cells and in human intestine by immunohistochmstry (7). We also demonstrated increased uptake of zinc by Xenopus laevis oocytes expressing the protein from injected in vitro synthesized ZnT5 mRNA indicative of a role in uptake of zinc across the plasma membrane (5). Kambe et al. (6), however, reported zinc uptake into Golgi-enriched vesicles prepared from HeLa cells expressing exogenous ZnT5 and have gone on to demonstrate a requirement for ZnT5, along with ZnT6 and ZnT7, for the activation in the Golgi apparatus of the zinc-requiring enzyme alkaline phosphatase (8, 9). Both groups detected two major transcripts of ZnT5 by Northern blotting, and comparison of the two published sequences shows that they differ at both the 5’ and 3’ ends, with the variant cloned by our group (referred to hereafter as variant B) being the shorter transcript. Alignment of both sequences with the human genome reveals that they are splice variants of the SLC30A5 gene (Fig. 1A), incorporating different exons at the 5’ and 3’ ends.

Many reports in the literature document the regulation of gene expression by zinc (5, 7, 10–16), including by DNA microarray hybridization (15, 16), and identify zinc transporter genes as among those regulated, both positively (e.g. SLC30A1 (ZnT1) (17)) and negatively (e.g. SLC39A4 (ZIP4)) (13), by increased zinc availability. The transcriptional up-regulation of gene expression by zinc through binding of the transcription
factor MTF1 to metal response elements (MREs)\(^2\) in gene promoter regions is well defined for the mouse metallothionein I and II genes (coding for small, cysteine-rich, intracellular zinc-binding proteins)\(^{18}\) and for mouse \(SLC30A1\) (ZnT1)\(^{19}\). Other zinc transporter gene promoters, including \(SLC30A5\), include consensus MRE sequences. A molecular mediator of zinc-induced transcriptional repression in mammalian systems has not yet been identified.

The current study investigated the regulation by zinc of the two cloned splice variants of the \(SLC30A5\) gene. We identify a common promoter region upstream of the first exon of variant A, which is transcriptionally repressed in response to both elevated and reduced extracellular zinc concentration, and demonstrate that a second mechanism, zinc-induced stabilization of ZnT5 mRNA, also contributes to the regulation of transcript abundance in response to zinc. We believe this is the first report of zinc-mediated zinc transporter regulation through effects on mRNA stability.

**EXPERIMENTAL PROCEDURES**

*Generation and Manipulation of Plasmid DNA Constructs—* The ZnT5 variant A-specific probe (198 bp) was generated by PCR from human genomic DNA using Thermo-Start DNA polymerase (ABgene) and primers and thermal cycling parameters as stated in Table 1. The product was subcloned into the vector pCR2.1-TOPO (Invitrogen), and identity to the required region was confirmed by sequencing (MWG Biotech). A large scale plasmid preparation (Plasmid Maxi kit, Qiagen) was digested with EcoRI, and the probe was purified from the band resolved by agarose gel electrophoresis using the QIAquick gel extraction kit (Qiagen).

Full-length cDNAs corresponding to ZnT5 splice variants A and B were generated by PCR or RT-PCR using KOD DNA polymerase (Novagen) with primers and thermal cycling parameters as stated in Table 1 and subcloned into the vector pCR2.1-TOPO (Invitrogen) to give plasmids pCR2.1-ZnT5A and pCR2.1-ZnT5B, respectively. The full-length cDNA corresponding to variant A was generated in three sections, which were spliced together. The template for the 5’ region was RNA from JAR cells. The template for the middle region was IMAGE clone 10213-g09. The template for the 3’ region was IMAGE clone 12254-j14. The middle and 3’ regions were spliced together in a PCR reaction using only the outermost primer pair and the product was then spliced to the 5’ region using the same strategy. The full-length cDNA corresponding to variant B was generated in two sections, which were spliced together. The template for the 5’ region was plasmid pZTL1-50 (5), and the template for the 3’ region was RNA from JAR cells. Both cDNAs were subcloned in-frame into the vector pEGFPN (Clontech), to give plasmids pEGFP-ZnT5A and pEGFP-ZnT5B, respectively, for expression as N-terminal fusion to GFP (SacI and ApaI for variant A and EcoRI for variant B).

Regions of genomic DNA sequence upstream of exons 1 (−2145 to +50 and −950 to +50, relative to the predicted start of transcription) and 3 (−2877 to +22 and −634 to +22, relative to the predicted start of transcription) of the \(SLC30A5\) gene were generated by PCR from human genomic DNA using Thermo-Start DNA polymerase (ABgene), Expand High Fidelity (Roche), or Advantage Genomic Polymerase Mix (Clontech). Primer pairs and thermal cycling parameters were as specified in Table 1, and products were subcloned into the vector pBlue-TOPO (Invitrogen). Correct insert orientation was confirmed by sequencing (MWG Biotech). Deletions to the 5’ end of the ZnT5 variant A promoter-reporter construct (−950 to +50) were made using the original promoter-reporter construct as template to generate PCR products, using Expand High Fidelity (Roche) and primers and thermal cycling conditions as listed in Table 1, which were then subcloned into the vector pBlue-TOPO. The MRE at position −410 to −404 (TGCACCTC) was mutated to a sequence different from the MRE consensus (CTGACTC) using the QuickChange Site-Directed Mutagenesis kit (Stratagene) and primers as listed in Table 1. The mutated construct was sequenced to confirm successful replacement of the MRE sequence. The element AAG-GCGAG at position −128 to −121 in the ZnT5 splice variant A promoter-reporter construct (−950 to +50) was deleted using a PCR-based method based on generating two overlapping products using ThermoStart DNA polymerase (ABgene) and primers spanning the region to be removed and incorporating the deletion. These PCR products were then joined in a third PCR reaction by including only the outer pair of primers, and the product was subcloned into the vector pBlue-TOPO. Correct insert orientation was confirmed by sequencing (MWG Biotech). The ZnT5 variant A region (−950 to +164) (including an MRE consensus sequence in the 5’-UTR) was generated from human genomic DNA using Expand High Fidelity (Roche) and primers and thermal cycling parameters as specified in Table 1 and subcloned into the vector pBlue-TOPO. Correct insert orientation was confirmed by sequencing (MWG Biotech). A human metallothionein 2a (MT2a) promoter-reporter construct, in the vector pBlue-TOPO, was generated as reported previously (10).

*Northern Blotting—* A multiple tissue human Northern blot was purchased from BD Biosciences. For Northern blots of Caco-2 mRNA, total RNA was prepared using TRIzol reagent (Invitrogen), and the poly-A+ fraction was purified using the NucleoTrap mRNA kit (Macherey-Nagel, Düren, Germany), following the manufacturer’s instructions. Electrophoresis (1 μg/lane) through 1% formaldehyde-containing agarose gels and capillary transfer to nylon membrane (Hybond N, Amersham Biosciences) was as described previously (5). Membranes were prehybridized for 20–30 min in Quickhyb (Statagene) before the addition of 25 ng of \(^{32}\)P-labeled probe (prepared using the Megaprime kit (Amersham Biosciences) and boiled for 2 min with 100 µl of salmon sperm DNA (100 ng/ml) before addition. Membranes were washed twice at 25 °C in 2× SSC (300 mM NaCl, 30 mM sodium citrate), 0.1% SDS, then once at 60 °C in 0.1× SSC, 0.1% SDS, and examined by autoradiography.
Subcellular Localization of GFP-tagged Constructs—For transfection into CHO cells, plasmids pEGFP-ZnT5A and pEGFP-ZnT5B were prepared using the EndoFree Plasmid Maxi kit (Qiagen). CHO cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 1% non-essential amino acids, 60 μg/ml gentamycin (all from Sigma) in 6-well plates on collagen-coated glass coverslips. Cells were transfected 24 h post-seeding, when 50% confluent, using GeneJammer transfection reagent (Stratagene), following the manufacturer’s instructions with a ratio of DNA to GeneJammer of 2 g:5 l. Twenty-four hours post-transfection, cells were fixed in 4% paraformaldehyde (in phosphate-buffered saline) for 30 min at room temperature, then washed three times with phosphate-buffered saline. In some experiments cells were treated with ZnCl2 or TPEN (at the indicated concentrations) for 1 h prior to fixation. Cells were permeabilized by treatment with 0.5% Triton X-100 for 15 min followed by a further phosphate-buffered saline wash. Cells were stained with 100 μM rhodamine-labeled wheat germ agglutinin for 5 min at room temperature, then visualized by laser scanning confocal microscopy using a Leica TCS NT confocal microscope.

**Reporter Gene Assay**—Caco-2 and JAR cells were cultured as described previously (5). For transfection, cells were seeded into 12-well plates at 3.5 × 10^5 cells/well and transfected 24 h after seeding with endotoxin-free preparations of reporter gene constructs (EndoFree Plasmid Maxi kit, Qiagen) using GeneJammer transfection reagent (Stratagene), following the manufacturer’s instructions and using a ratio of DNA to transfection reagent of 1.75 g:4.5 l. Transfection medium was removed after 24 h and replaced with serum-free medium containing 1.1 μM TPEN or 3, 100, or 150 μM zinc as ZnCl2. Cell lysates were prepared after 24 h and assayed for β-galactosidase activity using the substrate chlorophenol red-β-D-galactopyranoside, as described previously. Protein concentration of cell lysates was measured using the Bradford assay (Bio-Rad).

### TABLE 1

| Product | Primer sequences | Product size (bp) | Anneal. temp. (°C) | *Cycling parameters |
|---------|------------------|------------------|--------------------|---------------------|
| Variant A-specific probe | 5'-GACGCCTGTGTTGCTATGAGACAT | 188 | 55 | 30 s, 30 s, 90 s x 30 |
| Variant A cDNA – 5’ region | 5'-GGCTTATTTTCCCACTCCCACTCC | 189 | 55 | 30 s, 30 s, 90 s x 35 |
| Variant A cDNA – middle region | 5'-GGAGTTACCACTTCTCTGAGCAGT | 2032 | 52 | 30 s, 30 s, 90 s x 35 |
| Variant A cDNA – 3’ region | 5'-GACAGCTGCAATGAGAAGCAGT | 243 | 56 | 30 s, 30 s, 90 s x 35 |
| Variant A cDNA – 3’ + middle region | 5'-GGAGTTACCACTTCTCTGAGCAGT | 2157 | 60 | 30 s, 30 s, 3 min x 35 |
| *Full length variant A cDNA | 5'-GGAGTTACCACTTCTCTGAGCAGT | 2295 | 55 | 30 s, 30 s, 3 min x 35 |
| Variant B cDNA – 5’ region | 5'-GGAGTTACCACTTCTCTGAGCAGT | 1446 | 56 | 30 s, 30 s, 90 s x 35 |
| Variant B cDNA – 3’ region | 5'-GGAGTTACCACTTCTCTGAGCAGT | 286 | 53 | 30 s, 30 s, 90 s x 35 |
| *Full length variant B cDNA | 5'-GGAGTTACCACTTCTCTGAGCAGT | 1586 | 60 | 30 s, 2 min, 90 s x 5 | 30 s, 30 s, 90 s x 30 |
| Exon 1 upstream region-950 to +50 | 5'-GGACATAGAATCTTGGACAGTG | 1000 | 55 | 30 s, 30 s, 90 s x 30 |
| Exon 1 upstream region-2145 to +50 – first round | 5'-GGGATCTCTCTTCTCAGGCC | 3597 | 55 | 30 s, 30 s, 4 min x 30 |
| Exon 1 upstream region-2145 to +50 – nested | 5'-GGGATCTCTCTTCTCAGGCC | 2195 | 55 | 30 s, 30 s, 4 min x 30 |
| Exon 3 upstream region-634 to +22 | 5'-GGAAATGAGATCGATCC | 656 | 55 | 30 s, 30 s, 90 s x 30 |
| Exon 3 upstream region-2877 to +22 | 5'-GATCATGACGAGTGGGAG | 2899 | 55 | 30 s, 30 s, 4 min x 30 |
| Promoter construct-740 to +50 | 5'-GGTTCGCTTCATGCTCAGG | 790 | 55 | 30 s, 30 s, 90 s x 30 |
| Promoter construct-579 to +50 | 5'-GGTTGCTTGGAACAGCTCAC | 447 | 55 | 30 s, 30 s, 90 s x 30 |
| Promoter construct-397 to +50 | 5'-GGTTGCTTGGAACAGCTCAC | 204 | 55 | 30 s, 30 s, 90 s x 30 |
| Promoter construct-154 to +50 | 5'-GGTTGCTTGGAACAGCTCAC | 204 | 55 | 30 s, 30 s, 90 s x 30 |
| Promoter construct-950 to +50, minus MRE (5'-directed mutagenesis) | 5'-GGCTTTGGACTCAGCTGCGTC | 1000 | 55 | 30 s, 60 s, 8 min x 15 |
| Promoter construct-950 to +50, minus AcnMRE, 5’ region | 5'-GGTTGCTTGGAACAGCTCAC | 804 | 55 | 30 s, 30 s, 90 s x 30 |
| Promoter construct-950 to +50, minus AcnMRE, 3’ region | 5'-GGTTGCTTGGAACAGCTCAC | 169 | 55 | 30 s, 30 s, 90 s x 30 |
| Promoter construct-950 to +50, minus AcnMRE, full product | 5'-GGTTGCTTGGAACAGCTCAC | 973 | 55 | 30 s, 30 s, 90 s x 30 |
| Promoter construct-950 to +164 | 5'-GGTTGCTTGGAACAGCTCAC | 1114 | 55 | 30 s, 30 s, 90 s x 30 |
| ZnT5 (real-time analysis) | 5'-GGGTCGCTTCATGCTCAGG | 131 | 55 | 15 s, 20 s, 20 s x 35 |
| GAPDH (real-time analysis) | 5'-GGGTCGCTTCATGCTCAGG | 128 | 55 | 15 s, 20 s, 20 s x 35 |

* All PCR reactions included denaturation at 95 °C and extension at 72 °C and, when using the thermostart DNA polymerase enzyme (ABgene), KOD polymerase (Novagen), or *Pfu* (Stratagene QuikChange kit), an initial activation step of 95 °C for 15 min. Times are given in the order denaturing, annealing extension, and the number of cycles is indicated.

* Numbered according to GenBank™ sequence NM_022902.

* Numbered according to GenBank™ sequence AF393924.

* Numbered according to GenBank™ sequence NM_002046.
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Cell Viability Assay—Caco-2 cells were seeded into 96-well plates at 0.3 × 10⁶ cells/well. Culture medium was removed 48 h after seeding and replaced with 100 μl of serum-free medium containing 1.1 μM TPEN or 3 or 150 μM ZnCl₂. Cells were cultured for further 24 h, then 20 μl of CellTiter-Blue reagent (containing resazurin, which is reduced to resorufin by metabolically active cells; Promega) was added to each well. Plates were incubated at 37 °C for 6 h, and then absorbance was measured at 560 nm. Background absorbance (from wells without cells) was subtracted from all values.

Real-time RT-PCR—ZnT5 transcript abundance, in samples of total RNA prepared from Caco-2 cells, was measured by real-time PCR after DNase treatment (Promega; following the manufacturer’s instructions) and reverse transcription using Superscript III RNase H⁻ reverse transcriptase (Invitrogen; following the manufacturer’s instructions). Primers specific to ZnT5 and GAPDH (normalization control) were as listed in Table 1, and amplification was on a Roche Lightcycler 2.0 (Roche Applied Science) using Absolute QPCR SYBR Green Capillary mix (ABgene) using thermal cycling parameters as in Table 1. The amplification products from both primer pairs were sequenced (MWG Biotech) to confirm identity to the expected products.

RESULTS

We generated a probe specific to ZnT5 splice variant A (or at least to variants including the same 5’ exon) by including only exon 1 (nucleotides 22–219) and probed a human multiple tissue Northern blot, revealing a major hybridizing transcript of ~4 kb (Fig. 1B, i). Attempts to generate specific probes for regions unique to variant B were unsuccessful because of homology with other regions of the human genome, so we probed the same blot with a probe corresponding to the full-length variant B transcript, revealing two major transcripts, one corresponding exactly in position to that revealed by the variant A-specific probe and a second (slightly more abundant) transcript of ~3 kb. Levels of the transcripts differed between different tissues as already reported (6), and there were no obvious differences between tissues in the relative abundance of one transcript compared with the other.

Previous reports of the subcellular localization of ZnT5 were based on the use of antibodies with the potential to cross-react with both variants (6, 7). To confirm the reported difference in the pattern of subcellular localization of ZnT5 splice variants A and B, we expressed both transiently as N-terminally conjugates to GFP in the cell line CHO, selected because of the ease with which subcellular compartments could be viewed in these relatively cytoplasm-rich, flattened cells. Rhodamine-conjugated wheat germ agglutinin was used to stain the plasma membrane in unpermeabilized, paraformaldehyde-fixed cells and to stain the Golgi apparatus in cells permeabilized with Triton X-100 following paraformaldehyde fixation. GFP-tagged ZnT5 splice variant A showed co-localization with wheat germ agglutinin in permeabilized cells, consistent with localization to the Golgi apparatus (Fig. 2, A, C, D, and E), whereas the pattern of localization of splice variant B was more diffuse, including co-localization with wheat germ agglutinin staining in unpermeabilized cells, indicating some localization at the plasma membrane (Fig. 2B). For both splice variants, there was no alteration in the pattern of localization by changing the extracellular zinc concentration in either serum-free medium (1.1 μM TPEN, 3 μM ZnCl₂, 150 μM ZnCl₂) or medium containing 10% fetal calf serum (5 μM TPEN, 3 μM zinc, 100 μM ZnCl₂, 150 μM ZnCl₂) for the 1 h period immediately prior to viewing the cells (data not shown). We find no evidence for zinc-induced intracellular trafficking of either ZnT5 splice variant.

Based on the observation that the two alternative transcripts of ZnT5 incorporate different first exons, we hypothesized that transcription may be from different promoter regions, upstream of each first exon (Fig. 1A). To test this hypothesis we
subcloned genomic regions immediately upstream of the first exon of each splice variant into the reporter vector pBlue-TOPO (Invitrogen) and measured reporter gene (β-galactosidase) activity in both the human intestinal cell line Caco-2 and the human placental cell line JAR. Constructs including regions −950 to +50 and −2145 to +50 (relative to the end of the 5′-UTR) immediately upstream of the first exon of splice variant A showed reporter gene activity significantly elevated above negative control (vector minus putative promoter region) in both Caco-2 and JAR cells (Fig. 3A). In contrast, regions −2877 to +22 and −634 to +22 (relative to the end of the 5′-UTR) immediately upstream of the first exon of splice variant B (i.e. exon 3) were inactive in this respect (Fig. 3B). These observations indicate that both splice variants of the SLC30A5 gene are transcribed from a single promoter, upstream of the first exon of splice variant A.

To investigate transcriptional regulation by zinc of the SLC30A5 gene, we measured the response of the active promoter region to changes in the extracellular zinc concentration in Caco-2 cells, adding the zinc chelator TPEN to reduce extracellular zinc concentration to below that of the serum-free tissue culture medium (0.7–0.8 μM). Zinc was maximum at an extracellular zinc concentration of 3 μM ZnCl₂ to cells transfected with both the −950 to +50 and the −2145 to +50 promoter-reporter constructs for 24 h. Reporter gene activity was maximum at an extracellular zinc concentration of 3 μM and was reduced both by an increase in zinc concentration in a dose-responsive manner and by the addition of TPEN (Fig. 4, A and B). A potential effect on cell viability of TPEN at the concentration used (1.1 μM) and/or of 150 μM ZnCl₂, was excluded by showing that neither treatment affected the ability of cells to reduce resazurin to resorufin (Fig. 4A, inset (ii)). As a positive control, we carried out parallel experiments using the well characterized, zinc-responsive human MT2a promoter (−358 to +40) as the insert in the promoter-reporter construct, and as expected, reporter gene expression was increased at elevated zinc concentrations (Fig. 4A, inset (iii)). Mammalian zinc-responsive genomic elements responsible for transcriptional repression in response to elevated zinc concentration have not yet been identified so we sought to identify the region of the SLC30A5 promoter responsible for this effect. The promoter region −950 to +50 includes one metal response element consensus sequence (TGCRCNC) at position −410 to −404 (TGCACCT). Mutation of this region to a sequence no longer satisfying the core-consensus MRE motif (CTGACTC) had no measurable effect on the transcriptional response to zinc (Fig. 4A), allowing us to exclude this motif as mediating the zinc-induced transcriptional repression of the SLC30A5 promoter observed. We generated a series of progressive 5′ deletions of
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**A**

![Diagram](image)

**B**

![Diagram](image)

**FIGURE 4.** The response of the SLC30A5 promoter, and truncations and mutations thereof, to reduced and elevated extracellular zinc concentrations in Caco-2 cells. Promoter activity was detected as activity in cell lysates of a β-galactosidase reporter gene immediately downstream of the genomic regions (or mutations thereof) in the vector pBlue-TOPO (Invitrogen) assayed using the substrate chlorophenol red-β-D-galactopyranoside. Negative controls included vector only. A, shows relative levels of activity of regions of the promoter, including mutations, as indicated at extracellular zinc concentrations (3 and 150 μM) or in the presence of the zinc chelator TPEN (1.1 μM). "MRE core consensus sequence (TGCACTC) at −410 to −404 mutated (to CTGACTC)." Region −146 to −119, including promoter element reported to underlie the negative regulatory effect of zinc-humic acid on activity of the human mitochondrial aconitase promoter, deleted. Includes an additional MRE in the 5′-UTR (−950 to +164). Inset (i), cell viability measured by the conversion of resazurin to resorufin in the presence of TPEN (1.1 μM) and at extracellular zinc concentrations of 3 and 150 μM. Inset (ii), response of the human MT2a promoter (−358 to +40), assayed using the same reporter system, to manipulation of the extracellular zinc concentration in Caco-2 cells. Data are expressed as mean ± S.E. for n = 3–39. **, p < 0.01 compared with 3 μM zinc by Student’s t test or one-way ANOVA followed by Dunnett’s test. B, shows the response of regions of the SLC30A5 promoter of three different lengths (as indicated) to manipulation of the extracellular zinc concentration in Caco-2 cells. In this panel, data are normalized to activity at 3 μM zinc. All data are expressed as mean ± S.E. for n = 3–39. **, p < 0.01 compared with 3 μM zinc by one-way ANOVA followed by Dunnett’s test.

the SLC30A5 promoter region in the −950 to +50 promoter-reporter construct and observed that transcriptional repression in response to 150 μM zinc was retained in a construct including only the region −154 to +50, localizing the element responsible for zinc-induced transcriptional repression to this region. Removal of a further 38 nucleotides from the 5′ end of the promoter region (leaving nucleotides −116 to +50) resulted in a large reduction in promoter activity so that it was only slightly increased above negative control (data not shown). We identified an element on the antisense strand of the promoter (AAG-
Caco-2 cells, demonstrating an mRNA stabilizing effect of increased extracellular zinc concentration opposing the transcriptional response. The response of the MT2a transcript to increased extracellular zinc concentration in the presence of actinomycin D was attenuated dramatically (Fig. 5B), demonstrating efficacy of the actinomycin D treatment to block transcription.

As already noted, attempts to generate ZnT5 splice variant B-specific probes were unsuccessful, so we used real-time RT-PCR, using primers annealing to the region of sequence shared by both splice variants, to measure the effect of changes in extracellular zinc concentration, in the presence and absence of actinomycin D, on total ZnT5 transcript levels. The response to increasing extracellular zinc concentration and to inhibition of
transcription by the addition of actinomycin D had a similar profile as determined specifically for variant A by Northern blotting, reduced mRNA levels at elevated extracellular zinc concentration (150 μM but not 100 μM in this case) and increased ZnT5 transcript levels at the higher zinc concentrations in the presence of actinomycin D, demonstrating stabilization by zinc of ZnT5 mRNA.

**DISCUSSION**

We demonstrate that splice variants of the SLC30A5 gene are differentially localized in the cell and are transcribed from a single promoter region, which is negatively regulated both by increased and depleted extracellular zinc concentration, relative to standard cell culture conditions. The zinc-responsive element(s) mediating both regulatory effects is within the region −154 to +50, relative to the start of transcription. Importantly, we demonstrate, for what we believe to be the first time, zinc-mediated stabilization of a transcript involved directly in zinc transport/homeostasis.

To our knowledge, this is the first report demonstrating that splice variants of a zinc transporter gene occur at different subcellular localization, but evidence in the literature suggests that the existence of splice variants is not a phenomenon unique to ZnT5. For example, different laboratories, including our own, observed multiple bands or bands of different sizes in different tissues on Western blots using anti-ZnT1 antibodies (7, 10, 17, 21). The alternative localization of ZnT5 splice variants observed in the present study, in the Golgi apparatus (for variant A) and throughout the cell, including in the plasma membrane (variant B), are consistent with the two different patterns of localization and inferred roles in cellular zinc homeostasis reported previously, specifically delivery of zinc to zinc-activated apoenzymes in the Golgi apparatus, including homeostatic maintenance of secretory pathway function (variant A) (8, 9, 22) and uptake and/or efflux of zinc across the plasma membrane (variant B) (5).

Repressed zinc transporter expression in response to increased zinc availability has been reported previously, most notably for ZIP4 (13), but zinc-responsive promoter elements mediating such an effect and transcription factors binding to these elements have not yet been identified. The recent observation that the expression of Zip10, which includes MREs in the upstream region, is increased in the conditional MTF1 knockout mouse (23) indicates that MTF1 binding to MREs may mediate negative gene regulation, in addition to the well characterized transcriptional up-regulation. We have shown that mutation of the unique MRE at position −410 to −404 in the SLC30A5 promoter-reporter construct affected neither the transcriptional response to increased extracellular zinc nor that observed in the presence of the zinc chelator TPEN and so was responsible for neither effect. Analogous to the possible role of MTF1 in zinc-induced transcriptional repression, rather than activation, the *Saccharomyces cerevisiae* transcription factor ZAP1, which characteristically mediates transcriptional activation by binding to elements with the zinc-responsive element consensus sequence in gene promoter regions, has been found to mediate zinc-induced transcriptional repression through binding to a zinc-responsive element downstream of the TATA box in the ZRT2 gene (24). The SLC30A5 gene included an element with the MRE core consensus sequence in the 5′-UTR but we excluded this as mediating the zinc-induced transcriptional repression because this response was observed regardless of whether or not this region was included in the promoter region upstream of the reporter gene. Similarly, deletion of the promoter element reported to underlie the negative regulatory effect of zinc-humic acid on the activity of the human mitochondrial aconitase promoter (20) did not affect the profile of transcriptional regulation in response to zinc manipulation, so can be excluded as mediating these responses.

The magnitude of the response of the SLC30A5 promoter-reporter construct to increased extracellular zinc concentration was modest, consistent with observations on the magnitude of the regulatory responses of mammalian macronutrient and micronutrient transporters to variations in their substrate supply, including dipeptides (25), amino acids (26), and iron (27). Such responses are typically, although not without exception (e.g. Ref. 13), in the order of 1.5–2-fold. The magnitude of the measured transcriptional response to zinc is also commensurate with responses of zinc transporters, including ZnT5, to zinc availability observed in vivo (7, 10–13). Presumably, such modest changes in nutrient transporter expression are generally adequate to maintain intracellular and/or whole-body nutrient homeostasis within physiologically encountered variations in the nutrient supply.

As already noted, a particularly pertinent finding of the study was that ZnT5 mRNA was stabilized by increased extracellular zinc concentration. An earlier report in the literature identified zinc-induced mRNA stabilization of the labile c-fos and tristetrapolin mRNAs in TK-L cells (28). However, we are unaware of any previous evidence that effects on mRNA stability is a mechanism through which zinc regulates the expression of genes involved directly in zinc metabolism and/or homeostasis. The ability of iron to destabilize RNAs, most notably transferrin, with the iron response element in the 3′-UTR is one of the classical examples of homeostatic regulation of mRNA stability (reviewed in Ref. 29). The finding that regulatory mechanism acting in the opposite direction (i.e. substrate mRNA stabilization rather than destabilization) contributes to the regulation of zinc transport provides an exciting area for future research.

There are reports in the literature, including our own observations, of both increased and reduced ZnT5 expression in response to increased zinc availability (5, 7, 10, 29). The identification of these two mechanisms that can act in opposition provides a plausible explanation for such apparently divergent responses. It would appear that the net effect on ZnT5 expression of changes in zinc availability under specific conditions reflects a balance between transcriptional repression and mRNA stabilization. This finding, along with the fact that ZnT5, present in the cell as differentially localized splice variant, functions in association with other, presumably regulated, zinc transporters reveals a level of complexity to cellular zinc homeostasis greater than that already indicated by the presence of multiple zinc transporters with potentially overlapping functions.
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