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Topical Review

Sequence Analyses and Phylogenetic Characterization of the ZIP Family of Metal Ion Transport Proteins

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Abstract. Several novel but similar heavy metal ion transporters, Zrt1, Zrt2, Zip1-4 and Irt1, have recently been characterized. Zrt1, Zrt2 and Zip1-4 are probably zinc transporters in *Saccharomyces cerevisiae* and *Arabidopsis thaliana* whereas Irt1 appears to play a role in iron uptake in *A. thaliana.* The family of proteins including these functionally characterized transporters has been designated the Zrt- and Irt-related protein (ZIP) family. In this report, ZIP family proteins in the current databases were identified and multiply aligned, and a phylogenetic tree for the family was constructed. A family specific signature sequence was derived, and the available sequences were analyzed for residues of potential functional significance. A fully conserved intramembranous histidyl residue, present within a putative amphipathic, α-helical, transmembrane spanning segment, was identified which may serve as a part of an intrachannel heavy metal ion binding site. The occurrence of a proposed extramembranal metal binding motif (H X H X H) was examined in order to evaluate its potential functional significance for various members of the family. The computational analyses reported in this topical review should serve as a guide to future researchers interested in the structure-function relationships of ZIP family proteins.

Key words: Metal ion transporters — ZIP family — Iron — Zinc uptake — Plants — Animals — Yeast

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Introduction

Metal ions are required for the growth of all life forms. Since trace metals are frequently present in natural environments in exceedingly low amounts or in forms that are inaccessible, living organisms utilize high affinity, energy-coupled transport systems to accumulate these essential ions against tremendous concentration gradients (Guerinot, 1994; Silver, 1996). Because these ions are toxic when present in excess of the needs of the cell, living organisms have also evolved ion-specific efflux systems to protect themselves against their deleterious effects (Nies & Silver, 1995). Recent studies have shown that metal ion uptake and efflux are catalyzed by a plethora of transport systems belonging to a number of distinct protein families (Fagan & Saier, 1994; Saier et al., 1994; Paulsen & Saier, 1997; Saier, 1998*a*,*b*).

Eide et al. (1996) have characterized a novel iron regulated metal transporter gene (*IRT1*) from *Arabidopsis thaliana.* The *IRT1* gene complemented an iron uptake deficiency in *Saccharomyces cerevisiae,* and the uptake activity expressed in yeast cells was strongly inhibited by Cd^{2+}. The Irt1 protein was predicted to be an integral membrane protein with eight transmembrane α-helical segments (spanners) and an extramembranal metal binding sequence. In the native plant, the *IRT1* gene proved to be preferentially expressed in roots, and expression was induced by iron deficiency. The transporter exhibited altered regulation in plant lines bearing mutations that affected iron uptake (Eide et al., 1996).

One of the *IRT1* gene homologues, ZRT1 of *Saccharomyces cerevisiae,* proved to encode a high affinity zinc uptake system, induced at the transcriptional level

Table 1. Sequenced members of the ZIP family

Protein abbr.	Protein designation	Function	Organism	# Amino acids	# Putative TMSs	Accession #
Irt1 Ath	Iron-regulated metal transporter	Fe^{2+} uptake	*Arabidopsis thaliana*	339	8	gbU27590
Zip1 Ath	Zinc transporter	Zn^{2+} uptake	*Arabidopsis thaliana*	355	7 or 8	AF033535
Zip2 Ath	Zinc transporter	Zn^{2+} uptake	*Arabidopsis thaliana*	353	8	AF033536
Zip3 Ath	Zinc transporter	Zn^{2+} uptake	*Arabidopsis thaliana*	339	8 or 9	AF033537
Zip4 Ath	Zinc transporter	Zn^{2+} uptake	*Arabidopsis thaliana*	374	8	gbU95973
Zrt1 Sce	Zinc transporter	Zn^{2+} uptake	*Saccharomyces cerevisiae*	376	8	spP32804
Zrt2 Sce	Zinc transporter	Zn^{2+} uptake	*Saccharomyces cerevisiae*	422	8	pirS59319
GaiP Hsa	Growth arrest inducible protein		*Homo sapiens*	309	8	Entrez: 998569 Seq. id: 165867
Orf1 Hsa			*Homo sapiens*	429	7	gbD82060
Orf1 Mmu			*Mus musculus*	436	7	spQ31125
Orf1 Cel			*Caenorhabditis elegans*	223	5	gbZ70306
Orf2 Cel			*Caenorhabditis elegans*	274	6	gbU42437
Orf3 Cel			*Caenorhabditis elegans*	381	8	gbZ81110
Orf4 Cel			*Caenorhabditis elegans*	295	6	gbU28944
Orf5 Cel			*Caenorhabditis elegans*	477	8	gbU80447

by zinc deficiency (Zhao & Eide, 1996*a*). Like Irt1, Zrt1 is predicted to possess eight spanners as well as an extramembranal metal-binding domain. Zrt1 is one of two zinc uptake systems in *Saccharomyces cerevisiae*. The other, Zrt2, possesses lower affinity for Zn^{2+} and is not as highly regulated by Zn^{2+} availability (Zhao & Eide, 1996*b*). This low affinity Zn^{2+} uptake system, encoded by the *ZRT2* gene, is similar in sequence to Irt1 and Zrt1.

It has been noted that functionally uncharacterized homologues of Irt1, Zrt1, and Zrt2 are present in rice, trypanosomes, nematodes and humans (Eide et al., 1996; Zhao & Eide, 1996*a*,*b*; Grotz et al., 1998), but the phylogenetic relationships of these ORFs to the three functionally characterized members of the family cited above have not been determined. We therefore screened the databanks for potential homologous of Irt1, Zrt1, and Zrt2 and identified 15 members of the family. Several members of the family from *Arabidopsis thaliana* have recently been identified and functionally characterized, and a dendrogram of these proteins has appeared (Eide & Guerinot, 1997; Grotz et al., 1998). This family has been designated the Zrt/Irt-related protein (ZIP) family of metal ion transporters (Eide & Guerinot, 1997; Grotz et al., 1998).

In this topical review, we multiply align the available sequences, derive a family-specific signature sequence, and construct a phylogenetic tree. Members of the ZIP family appear to have widely divergent sizes, sequences and numbers of putative transmembrane α-helical segments. The proposed metal binding site (H X H X H), found in Irt1, Zrt1, and Zrt2 between spanners III and IV, is conserved in many but not all members of the family. The best conserved putative spanner (spanner IV) in the 15 ZIP family proteins analyzed is strongly amphipathic and possesses a central, fully conserved, histidyl residue on its hydrophilic side that could provide an intramembranous heavy metal ion binding site. While the proteins of the ZIP family are all predicted to exhibit specificity for metal ions, it is possible that they will prove to exhibit specificites for different cations, function with differing polarities and exhibit different subcellular locations.

Proteins of the ZIP Family

Table 1 lists the protein members of the ZIP family, provides the protein abbreviations to be used in this study, indicates their biological sources and functions when known, and provides accession numbers which allow easy access to the sequences (when available) as well as primary reference materials. Fifteen members of the family were identified, all of which were shown to be homologous to each other using the RDF2 (Pearson & Lipman, 1988) and GAP (Devereux, Hasberli & Smithies, 1984) programs with 500 random shuffles (Saier, 1996). These proteins vary in size from 223 to 477 amino acyl residues and possess from 5 to 8 putative transmembrane α-helical spanners (Table 1). Most of the variation in length occurs in their N-terminal regions. Thus, the five C-terminal putative spanners are present in all of the these proteins. Between eight and ten of the 15 proteins exhibit eight putative spanners, the maximal number observed for any family member. It is not known whether the smaller sizes and different apparent topological features represent true characteristics of these proteins or instead are attributable to sequencing artifacts.

Among the proteins listed in Table 1 are three new

ZIP family proteins. The genes encoding these proteins have recently been isolated from *Arabidopsis thaliana* by functional expression cloning in a Zn^{2+} uptake-deficient yeast strain (a *zrt1 zrt2* double mutant of *Saccharomyces cerevisiae*) (Grotz et al., 1998). These three proteins, designated Zip1, Zip2 and Zip3, show Zn^{2+} uptake activity when expressed in yeast with K_m values ranging from 2–14 μM. Based on competition assays, Zip2 (but not Zip1 or Zip3) is strongly inhibited by Cd^{2+} and Cu^{2+}. Zip1 and Zip3 are closely related to Irt1 while Zip2 is more distantly related (Grotz et al., 1998; *see below*). Zip1 and Zip3 are induced in the roots of Zn^{2+}-limited plants with little expression apparent in Zn^{2+} replete plants. Yet another *A. thaliana* ZIP family gene, *ZIP4*, was identified in the course of genome sequencing. *ZIP4* is also zinc-regulated, being expressed in both roots and shoots of zinc-deficient plants. No zinc or iron uptake activity was detected in yeast expressing *ZIP4*. These observations show that plants possess numerous ZIP family isoforms, several of which exhibit similar activities.

Multiple Alignment of ZIP Protein Sequences

A well conserved portion of the complete multiple alignment of the 15 currently recognized ZIP family proteins is shown in Fig. 1. As can be seen, Orf1 Mmu and Orf1 Hsa are almost identical. The region shown in Fig. 1 encompasses most of spanner IV and all of spanners V and VI according to the model of Zhao and Eide (1996*a*). Of particular note is the absence of gaps in the portion of transmembrane spanner IV shown, and the presence of fully conserved histidyl and glycyl residues in this spanner. Adjacent to and on the right hand side of the fully conserved intramembranous histidyl residue is found a semipolar or polar residue, usually a serine. Putative spanner IV proved to be strongly amphipathic when portrayed in helical wheel configuration with essentially all polar and semipolar residues localized to one side of the helix (*data not shown*). We predict that the histidyl residue and the adjacent (semi)polar residue in putative spanner IV comprise part of a heavy metal binding site in the center of the membrane. It is interesting to note that a fairly well conserved histidyl residue, substituted by polar or semipolar residues, and adjacent to another polar residue, is found at the beginning of spanner V. Spanners IV and V are thus proposed to in part comprise the transmembrane aqueous channel through which the substrate metal ion passes. It is noteworthy that all residues that appear in the consensus sequence are localized within or are immediately adjacent to the three putative spanners shown. We conclude that the assignments of spanners IV–VI (Eide et al., 1996; Zhao & Eide, 1996*a,b*) are likely to be largely correct (*see* Saier, 1994, 1996).

A Signature Sequence for the ZIP Family

The most conserved portions of the ZIP family proteins occur in and adjacent to spanner IV (Fig. 1). Based on the alignment shown in Fig. 1, a potential signature sequence for the ZIP family was derived. This sequence is:

[L I V F A] [G A S] [L I V M D] [L I V S C G] [L I V F A S] H [S A N] [L I V F A] [L I V F M A T] [L I V D E] G [L I V F] [S A N] [L I V F] [G S]

[Alternative residues at any one position are in brackets]

This sequence was screened against the SwissProt database. It retrieved only recognized members of the ZIP family. It is therefore a bona fide signature sequence (Bairoch, Bucher & Hofmann, 1997) and should prove useful for the identification of new ZIP family proteins as their sequences become available.

Putative Heavy Metal Binding Sequences

Eide et al. (1996) and Zhao and Eide (1996*a,b*) identified regions in the Irt1, Zrt1, and Zrt2 proteins that exhibit the sequence H X H X H between putative spanners III and IV. This sequence was postulated to serve as an extramembranal metal ion binding site in these proteins. Similar sequences have been identified in members of another heavy metal ion transport family, the cation diffusion facilitator (CDF) family (Paulsen & Saier, 1997 and *unpublished results*). In contrast to the $(H\ X)_n$ sequences found in some of the ZIP family proteins, $(H\ X)_n$ sequences in CDF family proteins occur between putative spanners IV and V with $n = 3–6$.

The complete multiple alignment of the ZIP family proteins (*not shown*) was examined to determine if the $(H\ X)_n$ sequences in the other sequenced members of the family were present at the site observed for Irt1, Zrt1, and Zrt2 or at other sites. Although it was not present in all of the ZIP family proteins at this site, at least one such sequence (or a close approximation to it) was found in all but two (Zip2 and Zip3) of the family members.

Table 2 summarizes the occurrence and sequences of $(H\ X)_3$ type putative metal binding sequences (or its variant in the case of Zrt2 Sce) found in 13 of the 15 ZIP family proteins. Their positions in these proteins are indicated. The five *C. elegans* homologues exhibit the postulated metal binding sequence near their C-termini, after the last spanner, in positions that, however, do not align with each other in the complete multiple alignment of their seuqences (*data not shown*). Some of these proteins were found to possess such a sequence in more than one location within their polypeptide chains. The 8 proteins listed in Table 2 that are derived from sources other than *C. elegans* do not exhibit this sequence within their

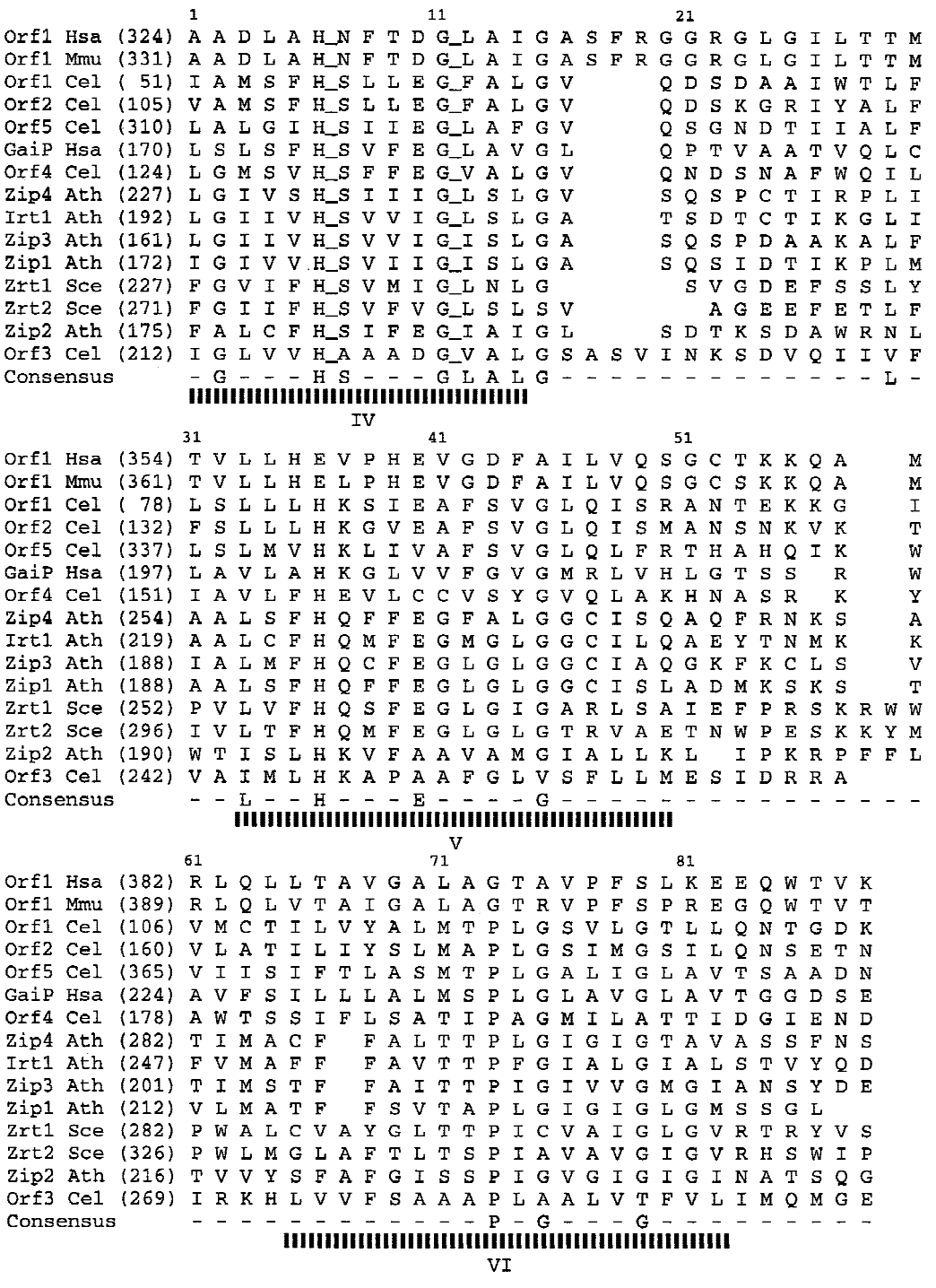

Fig. 1. Multiple alignment of a well-conserved region of fifteen sequenced members of the ZIP family. Protein abbreviations are as indicated in Table 1. The numbers in parentheses following the protein abbreviations correspond to the residue numbers of the first residue shown in each line. The alignment positions are indicated above the alignment, and the consensus sequence (8 of 15 residues at any one position conserved) is indicated below it. Below the consensus sequence, the positions of putative spanners IV, V and VI as suggested by Zhao and Eide (1996*a*,*b*) are shown. The degree of conservation within and to the left of putative spanner IV suggests that the GLALG sequence (*see* Consensus) may exhibit functional and/or structural significance. Fully conserved residues are indicated by a line adjacent to and to the right of the residue. The multiple alignment is based on the TREE program using a gap penalty of 8 (Feng & Doolittle, 1990), and it served as the basis for construction of the tree as shown in Fig. 2.

Table 2. Identification of putative metal ion binding sequence motifs in ZIP family proteins[a]

Protein abbreviation	Residue #	Sequence
Irt1 Ath	155	G H G H G H G
Zrt1 Sce	160	S H D H T H D
GaiP Hsa	149	L H S H G H L
Orf1 Hsa	142	G H G H S H G
Orf1 Mmu	142	G H G H S H G
Orf1 Cel	196	G H G H G H P
Orf2 Cel	264	E H G H Q H H
Orf3 Cel	70	V H Q H N H H
Orf4 Cel	245	G H G H S H N
Orf5 Cel	452	S H G H S H S
Zip1 Ath	142	G H V H I H T
Zip4 Ath	161	G H A H G H S
Zrt2 Sce	130	G H D H G D H

[a] The $(H X)_3$ motif could not be identified in Zip2 and Zip3.

C-terminal regions. Instead, the $(H X)_n$ sequence is found in their N-terminal or central regions preceding spanners IV–VI shown in Fig. 1. Most striking, however, was an extended histidine-rich region which constituted most of the first half of Orf1 Hsa or Orf1 Mmu. The human protein exhibits the following sequence:

(43) H G H S H R H S H E D F H H G H S H A H G H T H E S I W H G H T H D H D H G H S H E D L H H G H S H G Y S H E S L Y H R G H G H D H E H S H G − (90 residues) − H G H S H S − (32 residues) − H G H S H G H G H A H S H

All of this extensive region exhibiting the $(H X)_n$ pattern is not likely to be concerned with metal binding. It may instead represent an example of a reiterated sequence arising by repeated duplication of a short nucleotide sequence during evolution of these genes (Doolittle, 1989).

The sequence $(H X)_6$ was screened against the SwissProt database. It retrieved 61 proteins, 55 from eukaryotes and six from bacteria. The eukaryotic proteins serve a variety of functions (receptors, transcription factors, kinases, homeobox proteins, etc.) not obviously related to a biochemical function involving heavy metal ions. The prokaryotic proteins retrieved included the urease accessory proteins (UreE) of *Haemophilus influenzae* and *Yersinia enterocolitica*, the hydrogenase formation protein (HypB) of *Rhodobacter capsulatus*, and a probable FKBP-type peptidyl-prolyl cis/trans isomerase of *H. influenzae*. A characteristic feature of all functionally characterized proteins that were found to exhibit the $(H X)_6$ motif was that they require protein:protein interactions for their functions. Perhaps if the $(H X)_n$ motif has a function, it is to facilitate macromolecular interactions as is apparently true of certain other repeat sequences (Saier & McCaldon, 1988; Dagnall & Saier, 1997). Metal binding might facilitate these protein:protein interactions (Feese et al., 1994).

Phylogenetic Tree for ZIP Family Proteins

The phylogenetic tree for the ZIP family proteins, derived using the TREE program of Feng and Doolittle (1990), is shown in Fig. 2. The functionally characterized Zrt1 (Zhao & Eide, 1996*a*) and Zrt2 (Zhao & Eide, 1996*b*) proteins of *S. cerevisiae* cluster closely together as might be expected given their shared role in Zn^{2+} transport. Irt1 of *A. thaliana*, most likely involved in Fe^{2+} transport (Eide et al., 1996), and Zip4 Ath of unknown function, are the closest relatives of these two yeast proteins. Zip1, Zip3 and Zip2, all of *A. thaliana*, are increasingly more distant from Irt1 and Zip4. It is interesting to note that the yeast proteins as well as most of the plant proteins cluster together. Only Zip2 Ath is more distant from the other plant proteins than are the yeast proteins.

All other proteins represented are from animals, and they are all distantly related to the yeast and plant proteins. With the exceptions of Orfs 1 and 2 of *C. elegans*, these animal proteins are also distant from each other on the tree. However, GaiP Hsa and Orf5 Cel are about equally distant from each other and from Orf1 Cel and Orf2 Cel. Orf3 Cel and Orf1 Hsa are clearly the most distant members of the family, and the mouse homologue Orf1 Mmu is almost identical to Orf1 Hsa (*see* Fig. 1). Most of these animal proteins are likely to serve metal ion transport functions, but which ions they transport cannot be surmised. The fact that clustering is in general agreement with the phylogenetic kingdoms of the organisms of origin leads us to suggest that most of the paralogues of the ZIP family observed for any one kingdom arose after separation of the plant, animal and fungal kingdoms.

Conclusions and Perspectives

In Table 1, the sizes and numbers of putative spanners of the various ZIP family members are tabulated. Comparing the positions of these proteins on the phylogenetic tree shown in Fig. 2 with the numbers of spanners provided in Table 1 reveals that the two close yeast paralogues as well as most of the proteins from *A. thaliana*, which all cluster together on the right hand side of the tree, are full length proteins with about eight putative spanners. On the other hand, the tightly clustered Orfs 1 and 2 of *C. elegans* (Fig. 2) are the two smallest proteins represented, possessing 5 and 6 putative spanners, respectively (Table 1). The remaining proteins, which exhibit strikingly divergent sequences, as revealed by their distances from each other on the phylogenetic tree (Fig. 2), possess 6, 7 or 8 putative spanners. Whether or not these noteworthy size and topological differences represent characteristic features of these proteins or are merely artifactual differences due to inaccurate or in-

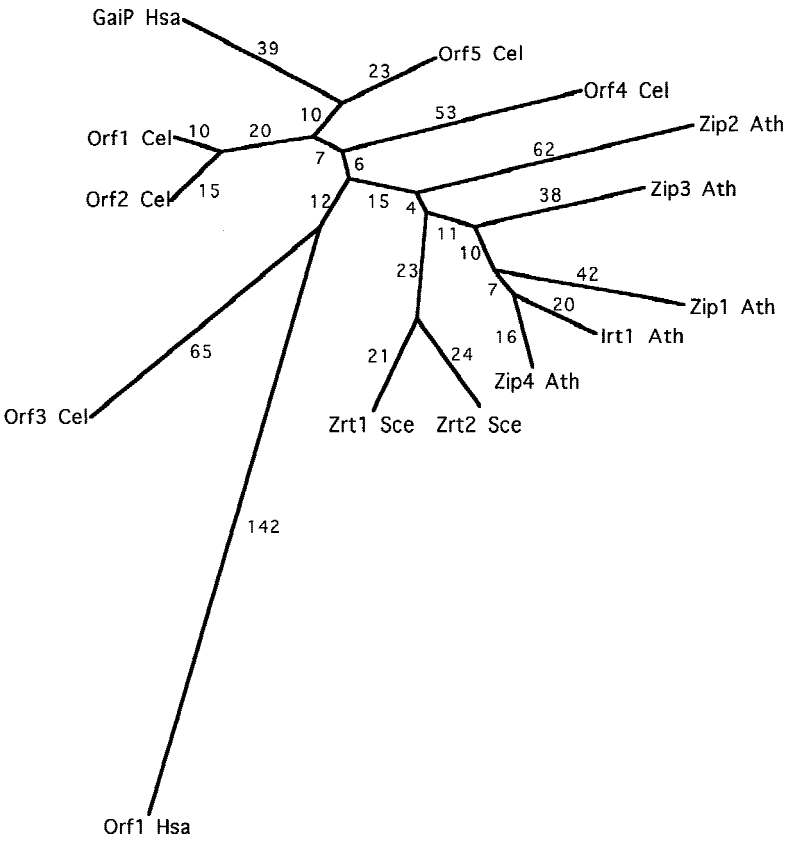

Fig. 2. Phylogenetic tree for the proteins of the ZIP family. Because Orf1 Hsa and Orf1 Mmu (Table 1) are nearly identical, only the former protein is shown. Protein abbreviations are as indicated in Table 1. Branch lengths are assumed to be approximately proportional to phylogenetic distances. Numerical values are in arbitrary units. The TREE program of Feng and Doolittle (1990) was used for tree construction and was based on the multiple alignment shown in Fig. 1.

complete sequencing, or to incorrect exon assignments, has yet to be determined.

The major findings resulting from the computational analyses presented in this report can be summarized as follows:

(i) All ZIP family proteins analyzed exhibit sequences corresponding to spanners IV–VIII in the functionally characterized Irt1, Zrt1, Zrt2 and Zip1, 2 and 3 proteins, but some or all of spanners I–III are lacking in some of the functionally uncharacterized homologues.

(ii) ZIP family proteins all possess fully conserved histidyl and glycyl residues in the most strongly amphipathic spanner, spanner IV. We postulate that this histidyl residue and the adjacent polar residue serve as parts of an intramembranous heavy metal binding site and aid in the lining of the transmembrane channel that provides the transport pathway. Residues in spanner V may also function in this capacity.

(iii) The proposed heavy metal binding sequence (H X H X H), found between spanners III and IV in Irt1, Zrt1, and Zrt2 (Eide et al., 1996; Zhao & Eide, 1996*a*,*b*), does not occur at this position in all members of the ZIP family. Although the five *C. elegans* homologues apparently lack the H X H X H sequence in the position corresponding to that found in Irt1 and Zrt1, they do possess this sequence in their C-terminal hydrophilic tails. Moreover, while the $(H\ X)_n$ sequence occurs in several ZIP family proteins in more than one place, Orf1 Hsa and the nearly identical Orf1 Mmu protein exhibit an extensive region of over 200 amino acyl residues near their N-termini in which the $(H\ X)_n$ motif occurs seven times with $n = 3$, 5, 6 or 7. Site-specific mutagenic analyses should allow definition of the functional significance of these sequences.

(iv) The phylogenetic analyses herein reported lead to specific as well as general functional predictions. Zrt1 and Zrt2 of *S. cerevisiae* provide very similar functions as Zn^{2+} transporters, and, therefore, Orfs 1 and 2 of *C. elegans*, as well as Irt1 and Zip4 of *A. thaliana* may also be expected to serve closely related functions. However, many of the uncharacterized homologues in animals are expected to serve quite dissimilar metal ion transport functions based on their widely divergent sizes, topologies and sequences.

(v) Finally, our studies serve to characterize the similarities and differences observed for 15 sequenced ZIP family proteins. These proteins are found only in eukaryotes, although they are found in at least three eukaryotic kingdoms, the fungal, plant and animal kingdoms. As an extensive body of prokaryotic sequence data, including the completely sequenced genomes of 14

bacteria and archaea are currently available for analysis, we tentatively suggest that the ZIP family arose in the eukaryotic kingdom after divergence of the three major kingdoms of life but before the eukaryotic phyla diverged from each other (Olsen, Woese & Overbeek, 1994). It is also possible that if prokaryotic proteins of the ZIP family exist, they have diverged in sequence from the eukaryotic sequences so that they are not recognizable. The high degree of sequence divergence observed for current members of the ZIP family is consistent with either of these proposals (Saier, 1994, 1996).

The availability of a signature sequence for the ZIP family should facilitate the identification of new family members as they become sequenced. The surprising size variability observed for the different currently sequenced members of the family warrants a careful reexamination of the nucleotide sequences from which the protein sequences were derived. We hope that our computational analyses will provide a guide for future studies of the structure-function relationships that characterize the proteins of the ZIP family.

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