

Characterization of Two Homologous Yeast Genes That Encode Mitochondrial Iron Transporters*

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Two different yeast genes were identified that when overexpressed suppressed the low iron growth defect of a mutation in the endoplasmic reticulum iron binding enzyme methyl sterol oxidase. These genes were determined to be novel and highly related. The deduced amino acid sequences indicated that both were membrane proteins having two identical histidine-rich motifs. The predicted proteins, while not ABC transporters, are homologous to a widely distributed family of transition metal transporters present in all kingdoms. Subcellular fractionation and fluorescence microscopy localized these gene products to mitochondria. Based on this result we term these genes *Mitochondrial Fe Transporters (MFT)*. Cells with disruptions in both genes show a growth defect on low iron medium, suggesting that these genes have redundant function and can affect cytosolic iron levels. Measurement of mitochondrial iron in cells grown in iron-rich medium overexpressing *MFT1* or *MFT2* show a 2–5-fold increase in iron compared with mitochondria from control cells. These results suggest that the mitochondria may act as a reservoir for iron that can be mobilized and used for cytosolic purposes.

zymosterol, an intermediate in yeast and mammalian sterol biosynthesis (6). The mutant *erg25* allele, termed *fet6-2*, has a missense mutation that appears to affect the ability of the protein to bind iron; the protein has normal activity in iron-replete medium but is nonfunctional in low iron medium. We screened for genes that when overexpressed could rescue the *fet6-2* mutant phenotype of poor growth on low iron medium. We report here the identification and characteristics of two homologous genes that suppress the mutant phenotype. We determined that the products of these genes affected the mutant protein indirectly by increasing cytosolic iron concentration. Subcellular fractionation studies localized the gene products to mitochondria and hence, we refer to them as *Mitochondrial Iron (Fe)Transporters (MFT)*. Disruption of either gene alone or in concert, however, did not affect viability or mitochondrial function. Disruption of both genes resulted in decreased growth in iron-limited medium. Incubation of cells that overexpress either gene in high iron medium resulted in an increase in mitochondrial iron. These genes appear to be members of an ancient and widely distributed family of transition metal transporters that function as transition metal resistance genes. Our data suggest that the *MFT* genes are involved with mitochondria iron movement and that the mitochondria may act as a reservoir for iron.

MATERIALS AND METHODS

S. cerevisiae and *Escherichia coli* Strains—The yeast strains employed in this study were derived from DY150 and DY1457 as described previously (7). The isolation and phenotype of the *fet6-2* strain is described in Li and Kaplan (5). The cells were grown in YPD (1.0% yeast extract, 0.2% peptone, 2.0% glucose), or in YPD made iron-limited by the addition of bathophenanthroline sulfonate (BPS)¹ (1), or in LIM medium, a synthetic medium that has defined concentrations of iron or other transition metals (8). Spheroplasts and a membrane preparation were obtained as described previously (5). Iron transport and ferrireductase activity was assayed as described in Eide *et al.* (8).

DNA Sequencing—DNA was sequenced using the dideoxy sequencing method using Sequenase from U. S. Biochemical Corp. and the Applied Biosystems automated sequencer. The primers were universal M13 primers and primers prepared using previously obtained sequencing data. Both strands were sequenced.

Identification and Subcloning of MFT1 and MFT2—DNA transformations of *E. coli* and *S. cerevisiae* were performed by standard procedures (9, 10). DNA fragments were isolated using Wizard Minipreps (Promega). The shuttle vector pTF63 was derived from YEplac195 and contained the Bluescript II polylinker (11). The Sau3A genomic library used in the complementation studies was described previously (5) and was the gift of Drs. D. Stillman and W. Ming. The high copy genomic library was transformed into *fet6-2* and colonies were isolated that complemented the growth defect on low iron medium. Plasmids were isolated and analyzed by restriction enzyme analysis. Two different complementing plasmids were sequenced using M13 primers. One plasmid (p20) contained a 3410-base pair segment of chromosome XIII,

In the past few years, enormous strides have been made in understanding the mechanisms that mediate iron accumulation in the budding yeast *Saccharomyces cerevisiae*. Genes for both high (1, 2) and low affinity plasma membrane (3) iron transport systems and their regulators (4) have been identified. While these studies have resulted in characterization of plasma membrane iron transport at the molecular level, much less is known about intracellular iron transport. Iron is distributed among different intracellular compartments: the vacuole in which iron may be stored, the mitochondria where the terminal steps of heme biosynthesis occurs, and the cytosol where many iron-utilizing enzymes are located. To date, little is known about the molecules or transporters responsible for intracellular iron movement.

We previously identified the yeast gene *ERG25* by a screen that, selected for mutants, showed poor growth on low iron medium (5). This gene encodes a methyl sterol oxidase that was deduced to be an oxo-diiron-containing enzyme based on its sequence and the observation that the mammalian methyl sterol oxidase requires iron for activity. This enzyme is responsible for an oxygen-mediated dimethylation of 4,4'-dimethyl

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¹ The abbreviations used are: BPS, bathophenanthroline sulfonate; YPD, yeast peptone dextrose; SNG, streptonigrin; ORF, open reading frame; PCR, polymerase chain reaction; GFP, green fluorescent protein.

while the other plasmid (p21) contained a 9094-base pair region of chromosome XVI. The only ORF that was identified on the p20 fragment was YMR177W. A number of different ORFs were present on the p21 plasmid. An ORF YPL224C was shown to be responsible for the complementation of *fet6-2* was proven by isolating the ORF using a *Hind*III site on the 5' end of the ORF and the polymerase chain reaction and primers on the 3' end of the ORF. The isolated ORF was subcloned into pTF63 and was shown to complement the low iron growth phenotype of *fet6-2*.

Construction of Deletion Strains—A double fusion PCR technique (12) was used for construction of $\Delta MFT1::URA3$ and $\Delta MFT2::HIS2$. The primers for amplification of the 5' end of $\Delta MFT1$ were: 5'-GCC CTG TAT AAA GAT CGT TC-3' and 5'-GTC GTG ACT GGG AAA ACC CTG GCG TTG TAG TCT CGT ATC GTG CC-3'. The primers for amplification of the 3' end of $\Delta MFT1$ were: 5'-TCC TGT GTG AAA TTG TTA TCC GCT TCC ACG CTA CCT AGA GAT AG-3' and 5'-GAG TAT TCG TAT GGT CGT GC-3'. The primers for amplification of the 5' end of $\Delta MFT2$ were: 5'-CTA TCA AGC AAT TCG GTT CC-3' and 5'-GTC GTG ACT GGG AAA ACC CTG GCG TCT GCT GTA CGT TCA GTG AC-3'. The primers for amplification of the 3' end of $\Delta MFT2$ were: 5'-TCC TGT GTG AAA TTG TTA TCC GCT TCT TAT CCT CAC TGA AGG AG-3' and 5'-CAT CAA CAA ACT CGA CGT CC-3'. The *URA3* gene was used as the selectable marker for the $\Delta MFT1$ disruptant while the *HIS3* gene was used as the selectable marker for the $\Delta MFT2$ disruptant (12). The PCR conditions for the 5' end and 3'-end-flanking sequences of two gene deletions were: 0.5 mM primer mixture, 3 mM Mg, denaturing at 94 °C, annealing at 50 °C, elongating at 70 °C, 20 s for 35 cycles in Idaho Technology Air Thermo-Cycler. The PCR conditions for the first fusion of the upstream fragment to the marker and the second fusion to the downstream fragment were performed in Perkin Elmer PCR machine at conditions of denaturing 94 °C, 40 s, annealing 60 °C, 40 s, elongating 72 °C, 3 min, for 35 cycles.

The purified double fusion PCR products were transformed into a wild-type diploid strain DY1640. After sporulation and dissection, spores containing the disruption were selected either by growth on Ura⁻ or His⁻ media. Correct integration was tested by colony PCR and Southern blotting.

Construction of Epitope-tagged and Green Fluorescent Protein (GFP) Fusion Protein—Both MFT1p and MFT2p were epitope-tagged with MYC at their carboxyl terminus using the polymerase chain reaction. For MFT1p, an 180-base pair fragment that encompassed a unique *Bsu*36I restriction site to the 3' end of the gene was amplified using two primers: 5'-CGT TAC TGT CCT CAG GAC CGA ATT TAC GCG-3' and 5'-AAG GAA AAA AGC GGC CGC TTA ATT CAA GTC CTC TTC AGA AAT GAG CTT TTG CTC CAT AAT ATG AGT ATT CGT ATG GTC-3'. The 3' primer included a MYC epitope. For MFT2p, two primers were used to make a fragment which included a *Bcl*I site and a MYC epitope at the 3' end of the ORF. The primers were: 5'-GGC TTG TAG TGT CAG GTT TGA TCA TAA AAA CAG GTG GAC-3' and 5'-AAG GAA AAA AGC GGC CGC TTA ATT CAA GTC CTC TTC AGA AAT GAG CTT TTG CTC CAT ATT TTT ATG GGT GTG AGT ATC GGC-3'. The PCR conditions were the same as described above using the Idaho Technology thermal cycler. Each construct was ligated into its cleavage site in the 3'-terminus of the plasmid. The plasmids were tested for their ability to complement the low iron growth defect of *fet6-2*.

The GFP was fused to the carboxyl terminus of MFT1p and MFT2p as described by Niedenthal *et al.* (13). A full gene sequence of *MFT1* was made by the polymerase chain reaction and inserted into the MET promoter containing plasmid pGFP-C-FUS at the *Xba*I and *Hind*III sites. The primers were: 5'-TGC TCT AGA TAT ATA TTA TTG CAT CAC ACA AAC ATC GCT-3' and 5'-CCC AAG CTT AAT ATG AGT ATT CGT ATG GTC-3'. The conditions for the polymerase chain reaction were as described above for Perkin Elmer machine. The plasmid containing the *MFT1-GFP* fusion construct was transformed into *fet6-2* and could complement the low iron growth defect.

Subcellular Fractionation—Mitochondrial isolation was performed as described by Glick and Pons (14). Briefly, yeast cells transformed with pTF63(*MFT1* Myc) were grown in CM-ura-glycerol-ethanol medium to an A_{600} of 3.0. The cells were collected by centrifugation, washed and spheroplasts were obtained using oxalyticase digestion. The spheroplasts were homogenized using a Dounce homogenizer and a crude mitochondrial fraction isolated by centrifugation. The mitochondrial fraction was loaded to the top of a 15% Percoll gradient and centrifuged at $59,000 \times g$ for 27 min in a Ti75 rotor.

For Western analysis, samples of yeast or fractions from the Percoll gradient were run on SDS-polyacrylamide gel electrophoresis in the presence of β -mercaptoethanol (15). The gels were transferred to nitrocellulose, and the nitrocellulose was blocked overnight at 4 °C by the

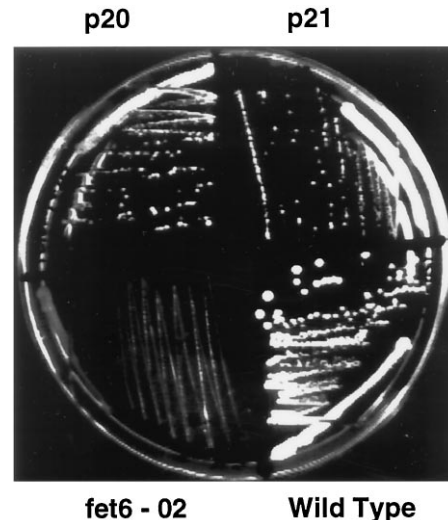


FIG. 1. **Partial complementation of *fet6-2* by p20 and p21.** Cells were streaked on iron-limited medium YPD (BPS5). Wild type cells; *fet6-2* transformed with p20; *fet6-2* transformed with p21; *fet6-2*. Note that transformation with p20 or p21 resulted in only partial complementation of the low growth phenotype of *fet6-2*.

addition of 5.0% non-fat dry milk dissolved in 20 mM Tris-HCl, 0.9% NaCl, 0.1% Tween 20 (pH 8.0). The blots were incubated with the primary antibody for 20 min at 37 °C, washed and incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Jackson Immuno Research Laboratories Inc.) for 1 h at room temperature. The filters were washed and developed using the ECL procedure (Amersham Life Science) as per the manufacturer's instructions. Samples were treated with endoglycosidase H according to the manufacturer's instructions (New England Biolabs).

Immunofluorescence—Cells in log phase were harvested and reincubated in 3.7% formaldehyde for 1 h at 30 °C and then resuspended in fixative buffer that contained 4.0% paraformaldehyde (pH 6.5) overnight at 30 °C. The fixed cells were then washed and treated with 200 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.0% β -mercaptoethanol for 10 min and then incubated with 1 mg/ml oxalyticase. The cells were permeabilized by the addition of 2.0% SDS in 1.2 M sorbitol for 2 min, washed in sorbitol buffer, and then allowed to settle on poly-L-lysine treated coverslips for 30 min. The cells were washed three times with 0.5% bovine serum albumin-phosphate-buffered saline and then incubated with a 1:500 monoclonal mouse anti-Myc antibody for 10 h. The coverslips were washed and then incubated with a 1:200 dilution of a Texas Red-conjugated goat anti-mouse antibody (Molecular Probes) for 2 h at room temperature. The cells were visualized using a Nikon inverted fluorescence microscope with a Zeiss 100 \times oil immersion objective. Images (512 \times 512) were acquired using a Photometrics cooled CCD camera and a Macintosh workstation running OncorImage 3-D cytometry software. A multidye filter set was used in which excitation filters of 575 nm (Texas Red) were selected from a computer-controlled filter wheel in conjunction with a multi-wavelength emitter and dichroic filter set (640 nm; XF56 set from Omega Optical). Out-of-focus blur was removed by adjacent plane fast Fourier deconvolution using the inverse of the modulation transfer function of the microscope. Adjacent planes were taken at 0.5- μ m intervals under control of the OncorImage software package. Prior to deconvolution, all images were corrected for background and flatfield. Images were then scaled to 256 levels of gray before output to a film recorder.

RESULTS

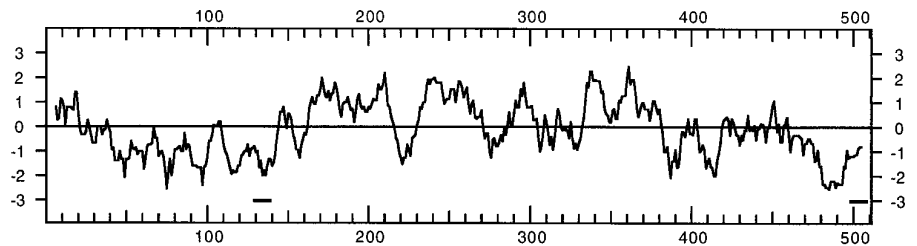
Identification of *MFT1* and *MFT2*—Incubation of *fet6-2*, an *erg25* mutant, in low iron medium results in defective sterol synthesis leading to a marked growth deficit (5). To isolate extragenic suppressors, *fet6-2* was transformed with a high copy yeast genomic library and colonies that demonstrated enhanced growth on low iron medium were isolated. After screening 20,000 colonies, a plasmid was isolated (p20) that allowed partial complementation of the *fet6-2* growth defect on iron limited medium (Fig. 1). The gene responsible for the low iron growth enhancement was identified by subcloning as ORF

A

YMR177W	1	MLR	ICVKR	PCIKIV	LSQ	VRPALL	V	RKENL	HIST	33																									
YPL224C	1	MLR	ISIDS	- - - - -	IKQ	FGS	FVTG	YNNTS	YHAA	27																									
YMR177W	34	G	VKVEK	SS	I	INQKDP	NKVR	VEIN	ELKR	QAEIEK	66																								
YPL224C	28	G	RAIRT	SSL	LYS	- - - - -	TMISA	N	P	RRCLHSSK	53																								
YMR177W	67	AA	I	KE	LEK	NPQY	QKL	AEAFN	SHDH	VHLRES	ETEE	99																							
YPL224C	54	LLN	KE	EGQE	EGYNE	QL	ISKMS	S	QNGS	NSRQN	ES	86																							
YMR177W	100	QND	I	ISL	G	T	IRDY	KSS	KCE	QADKP	SSLN	LHSH	132																						
YPL224C	87	-	GK	- - - - -	-	-	KEGK	AS	- - - - -	-	SVKS	L	LQHT	103																					
YMR177W	133	HS	HGH	THS	HA	AH	NPLL	V	LS	TE	QIR	KNA	GV	RITW	165																				
YPL224C	104	HS	HS	H	TH	MH	-	-	DN	NPLL	S	LN	V	VQ	QIR	KNA	PG	VR	ITW	134															
YMR177W	166	V	GL	G	V	N	V	G	I	A	I	G	K	F	F	G	G	I	V	F	H	S	Q	A	L	F	A	D	A	I	H	A	I	198	
YPL224C	135	I	GL	A	S	N	V	G	M	A	V	G	K	F	V	G	G	I	T	F	H	S	Q	A	L	L	A	D	S	V	H	A	L	167	
YMR177W	199	S	DM	V	S	D	L	L	T	L	L	S	V	G	L	A	A	N	K	P	T	A	D	Y	P	Y	G	Y	G	K	I	E	T	231	
YPL224C	168	S	D	L	V	S	D	F	L	T	L	F	S	V	Q	Y	A	S	R	K	P	T	S	E	Y	P	Y	G	Y	G	K	V	E	T	200
YMR177W	232	V	G	S	L	A	V	S	T	I	L	A	M	A	G	I	S	I	G	W	S	S	L	C	A	L	V	G	P	V	I	P	H	T	264
YPL224C	201	V	G	S	L	A	V	S	T	I	L	A	M	A	G	I	S	I	G	W	S	S	L	C	A	I	V	G	P	V	I	P	H	A	233
YMR177W	265	I	I	D	T	I	G	-	N	L	G	H	A	H	T	Y	S	E	D	I	I	E	D	V	T	D	I	N	A	A	W	I	A	A	296
YPL224C	234	I	L	E	S	M	A	G	L	I	G	E	T	H	S	H	S	Q	S	L	T	Q	Q	A	T	N	V	N	A	V	W	I	A	A	266
YMR177W	297	A	S	I	A	A	K	E	W	I	F	R	A	T	R	K	I	A	I	N	T	N	S	N	V	L	M	A	N	A	W	H	H	R	329
YPL224C	267	G	S	I	L	V	K	E	W	V	F	Q	A	T	K	K	V	A	I	Q	T	N	S	N	V	L	M	A	N	A	W	H	H	R	299
YMR177W	330	V	D	S	L	T	S	L	V	A	L	V	A	I	S	T	G	Y	L	V	N	I	Q	S	L	D	T	I	G	G	L	I	V	S	362
YPL224C	300	V	D	S	L	T	S	L	V	A	L	V	A	I	T	S	S	Y	F	F	N	I	Q	S	L	D	N	L	G	G	L	V	V	S	332
YMR177W	363	G	L	I	I	K	A	G	G	E	G	M	C	I	A	I	K	E	L	I	D	Q	S	V	S	R	D	D	P	R	Y	L	E	I	395
YPL224C	333	G	L	I	I	K	T	G	G	Q	G	I	L	S	S	L	K	E	L	V	D	Q	S	I	P	P	T	D	P	R	Y	L	E	I	365
YMR177W	396	E	T	L	V	K	D	T	L	N	K	L	I	S	N	N	S	Q	K	P	Y	G	L	K	E	L	T	L	L	S	S	G	P	428	
YPL224C	366	E	S	V	I	K	D	S	I	G	S	L	K	T	D	L	D	L	K	Q	S	L	H	V	R	D	L	T	I	L	A	S	G	P	398
YMR177W	429	N	L	R	G	H	L	T	L	E	V	P	L	Q	K	W	G	N	I	L	G	V	N	E	F	E	I	V	T	H	H	L	R	N	461
YPL224C	399	N	L	R	A	T	T	T	L	E	V	P	V	L	H	S	G	Q	E	V	G	I	R	F	L	E	N	A	I	S	T	I	R	E	431
YMR177W	462	V	L	T	N	E	V	S	N	L	R	R	L	D	I	E	Y	V	E	E	K	N	-	-	G	-	E	E	N	E	H	I	489		
YPL224C	432	D	L	R	M	K	V	P	N	V	G	K	V	D	V	E	F	V	D	V	T	S	D	S	K	G	D	L	E	H	S	H	D	T	464
YMR177W	490	K	G	Q	Q	N	Y	K	E	D	V	L	I	K	H	D	H	T	N	T	H	I													510
YPL224C	465	K	S	T	N	H	T	H	T	H	S	D	S	A	D	T	H	T	H	K	H	-													484

FIG. 2. Deduced amino acid sequence of the complementing open reading frames in P20 and P21. Subcloning experiments localized the complementing activity of p20 to open reading frame YMR177W of chromosome 13 and of p21 to open reading frame YPL224C on chromosome 16. A, conceptual translation of YMR177W and YPL224C. B, Kyte-Doolittle hydropathy analysis (16) of the amino acid sequence of YMR177W and YPL224C. The underlined sequences are the histidine-rich domains.

B



YMR177W. This gene when placed in a multicopy plasmid, could by itself suppress the low iron growth deficit of *fet6-2*. The gene, however, when placed in a centromeric plasmid was unable to rescue the low iron growth phenotype. Using the same library a different plasmid (p21) was isolated that also complemented the low iron growth phenotype of *fet6-2*. Subcloning determined that the responsible ORF, YPL224C, when expressed from a multicopy but not a single copy plasmid, suppressed the *fet6-2* low iron growth defect.

Examination of the deduced amino acid sequence of these two genes revealed that they are highly homologous (52% iden-

tity and 75% similarity) and novel (Fig. 2). They appear to encode membrane proteins with six putative transmembrane domains. The sequences show no identifiable leader or endoplasmic reticulum retention signal. Prosite analysis of either protein suggests that the amino terminus could be a mitochondrial targeting sequence. There is, at best, one potential *N*-glycosylation site. A feature of interest is two conserved histidine-rich clusters found in both proteins. In particular, the sequence HTHSHXHTXHX strongly suggests a metal binding motif.

These genes show extensive homology to other genes identi-

MFT1	1	MLR	ICV	KRPC	I	K	I	V	L	S	Q	V	R	P	A	L	L	V	R	K	E	N	L	H	I	S	T	33								
MFT2	1	MLR	I	S	I	D	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	27								
ZnT-2	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2								
ZnT-3	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	19								
C.elegans	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13								
E.Coli	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0								
CZCD	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0								
COT1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0								
ZRC1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0								
MFT1	34	G	V	K	V	E	K	S	S	I	N	Q	K	D	P	N	K	V	R	V	E	I	N	E	L	K	R	Q	A	E	I	E	K	66		
MFT2	28	G	R	A	I	R	T	S	S	L	Y	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	53		
ZnT-2	3	S	R	S	F	F	G	A	L	W	K	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15		
ZnT-3	20	D	R	G	G	A	G	G	S	L	R	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32		
C.elegans	14	S	C	S	L	W	G	C	H	N	K	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	26		
E.Coli	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0		
CZCD	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0		
COT1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0		
ZRC1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0		
MFT1	67	A	A	I	K	E	L	E	K	N	P	Q	Y	Q	K	L	A	E	A	F	N	S	H	D	H	V	H	L	R	E	S	E	T	E	99	
MFT2	54	L	L	N	K	E	G	Q	E	E	G	Y	N	E	Q	L	I	S	K	M	S	S	Q	N	G	S	N	S	R	Q	N	E	S	E	86	
ZnT-2	16	S	R	I	P	P	V	N	-	L	P	S	V	E	L	A	V	Q	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32	
ZnT-3	33	L	F	T	E	P	S	E	P	L	P	E	E	S	K	P	V	E	M	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	51	
C.elegans	27	A	Q	N	P	Q	C	T	R	V	P	H	I	Y	N	T	D	E	G	R	K	T	T	-	-	-	-	-	-	-	-	-	-	-	48	
E.Coli	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	
CZCD	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	
COT1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	
ZRC1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	
MFT1	100	Q	N	D	I	I	S	L	G	T	I	R	D	Y	K	S	S	K	C	E	Q	A	D	K	P	S	S	L	N	L	H	S	H	T	132	
MFT2	87	-	G	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	103	
ZnT-2	33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	36	
ZnT-3	52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	55	
C.elegans	49	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	56	
E.Coli	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	
CZCD	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	
COT1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
ZRC1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
MFT1	133	H	S	H	G	H	T	H	S	A	A	H	N	P	L	L	V	L	S	T	E	Q	I	R	K	N	A	G	V	R	I	T	W	165		
MFT2	104	H	S	H	S	H	T	H	M	H	-	-	D	N	P	L	L	S	L	N	V	Q	I	K	K	N	P	G	V	R	I	T	W	134		
ZnT-2	37	H	A	Q	K	D	S	G	S	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	58	
ZnT-3	56	H	R	D	P	L	P	P	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	77	
C.elegans	57	H	D	E	A	D	S	T	D	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	77	
E.Coli	5	H	S	H	T	S	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	22	
CZCD	5	H	S	H	D	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	18	
COT1	1	M	K	L	G	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11	
ZRC1	2	I	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	
MFT1	166	V	G	L	G	V	N	V	G	I	A	I	G	K	F	F	G	G	I	V	F	H	S	Q	A	L	F	A	D	A	I	H	A	I	198	
MFT2	135	I	G	L	A	S	N	V	G	M	A	V	G	K	F	V	G	G	I	T	F	H	S	Q	A	L	A	D	S	V	H	A	L	167		
ZnT-2	59	V	A	S	A	I	C	L	V	F	M	I	G	E	I	I	G	G	Y	L	A	Q	S	L	A	I	M	T	D	A	A	H	L	L	91	
ZnT-3	78	A	A	C	A	V	C	F	V	F	M	A	G	E	V	V	G	G	Y	L	A	H	S	L	A	I	M	T	D	A	A	H	L	L	110	
C.elegans	78	L	T	V	V	L	C	L	F	F	M	V	C	E	V	I	G	G	V	L	A	G	S	L	A	I	V	T	D	A	A	H	L	L	110	
E.Coli	23	Y	A	F	G	V	T	A	G	F	M	L	V	E	V	V	G	G	F	L	S	G	S	L	A	L	A	D	A	G	H	M	L	55		
CZCD	19	I	A	L	A	L	T	G	T	F	L	I	A	E	V	V	G	G	V	M	T	K	S	L	A	L	I	S	D	A	H	M	L	51		
COT1	12	S	L	L	L	D	T	V	F	F	G	I	E	I	T	T	G	Y	L	S	H	S	L	A	L	I	A	D	S	F	H	M	L	44		
ZRC1	11	S	L	L	T	L	D	T	V	F	F	L	L	E	I	T	I	G	Y	M	S	H	S	L	A	L	I	A	D	S	F	H	M	L	43	
MFT1	199	S	D	M	V	S	D	L	L	T	L	S	V	G	L	A	A	-	N	K	P	T	A	D	Y	P	Y	G	Y	G	K	I	E	230		
MFT2	168	S	D	L	V	S	D	F	L	T	L	F	S	V	Q	Y	A	S	-	R	K	P	T	S	E	Y	P	Y	G	Y	G	K	V	E	199	
ZnT-2	92	T	D	F	A	S	M	L	I	S	L	F	S	L	W	V	S	-	R	P	A	T	K	T	M	N	F	G	W	Q	R	A	E	123		
ZnT-3	111	A	D	V	G	S	M	M	G	S	L	F	S	L	W	L	S	T	-	R	P	A	T	R	T	M	T	F	G	W	H	R	S	E	142	
C.elegans	111	T	D	F	A	S	V	L	I	S	L	F	S	L	Y	I	A	R	-	R	P	P	S	Q	K	M	S	F	G	F	H	R	A	E	142	
E.Coli	56	T	D	T	A	A	L	L	F	A	L	L	A	V	Q	F	S	R	-	R	P	P	T	I	R	H	T	F	G	W	L	R	L	T	87	
CZCD	52	T	D	T	V	A	L	A	I	A	L	A	A	I	A	I	A	K	-	R	P	A	D	K	K	R	T	F	G	Y	R	F	E	83		
COT1	45	N	D	I	I	S	L	V	V	A	L	W	A	V	N	V	A	K	N	R	N	P	D	S	T	Y	T	Y	G	W	K	R	A	E	77	
ZRC1	44	N	D	I	I	S	L	V	V	A	L	W	A	V	D	V	A	K	N	R	G	P	D	A	K	Y	T	Y	G	W	K	R	A	E	76	

FIG. 3. Homology of YMR177W and YPL224C to other transition metal transporters. These sequences were compiled using Blast analysis and the cluster W algorithm. For reasons described below, we refer to YMR177W as *MFT1* and YPL224C as *MFT2*. *COT1* is a cobalt resistance gene found in *S. cerevisiae* (17), *ZNT2* (18), and *ZNT3* (19) are mammalian zinc transporters, *czcd* is a cobalt, zinc, cadmium resistance gene found in *A. eutrophus*, a Gram-negative bacteria (20). The *E. coli* sequence (f313, accession no. 1786966) and the *Caenorhabditis elegans* sequence (T18d3.3, accession no. 1082146) were the result of genome sequencing projects.

fied as transition metal transporters, particularly for zinc, cadmium, or cobalt. These genes are found in diverse species, including bacteria, yeast, and mammals. These transporters have been localized to different organelles; mitochondria, endosomes, and plasma membrane (Fig. 3). For example Cot1p, which is involved in cobalt resistance in yeast is mitochondrial (17). The mammalian zinc transporters, *ZNT2* and *ZNT3*, are

MFT1	231	T V G S L A V S T I L A M A G I S I G W S S L C A L V G P V I P H	263
MFT2	200	T V G S L A V S T I L A M A G I S I G W S S L C A I V G P V I P H	232
ZnT-2	124	I L G A L L S V L S I W V V T G V L V Y L A V Q R L I S G - - D Y	154
ZnT-3	143	T L G A L A S V V S L W M V T G I L L Y L A F V R L L H S - - D Y	173
C.elegans	143	V L G A F F S V F L I W I V T G V L V V L A I M R I V S G - - D Y	173
E.Coli	88	T L A A F V N A I A L V V I T I L I V W E A I E R F R T - - P R	117
CZCD	84	I L A A A F N A L L L F G V A I Y I L Y E A Y L R L K S - - P P	113
COT1	78	I L G A L I N A V F L I A L C V S I L I E A L Q R I I A P - - P V	108
ZRC1	77	I L G A L I N A V F L I A L C F S I M I E A L Q R L I E P - - Q E	107
MFT1	264	T I I D T I G - N L G H A H T Y S E D I I E D V T D I N A A W I A	295
MFT2	233	A I L E S M A G L I G E T H S H S Q S L T Q Q A T N V N A V W I A	265
ZnT-2	155	E I K G D T M L I T S G C A V A V N I I M G L A L H Q S G - - - -	183
ZnT-3	174	H I E G G A M L L T A S I A V C A N L L M A F V L H Q A G - - - -	202
C.elegans	174	E V E G G I M A L T A A L G V V V N L V M L A L L Y F G G - - - -	202
E.Coli	118	P V E G G M M M A I A V A G L L A N I L S F W L L H H G S - - - -	146
CZCD	114	Q I E S T G M F V V A V L G L I I N L I S M R M L S S G - - - -	141
COT1	109	I E N P K F V L Y V G V A G L I S N T V G L F L F H D N - - D Q E	139
ZRC1	108	I Q N P R L V L Y V G V A G L I S N V V G L F L F H D H G S D S L	140
MFT1	296	A A S I A A K E W I F R - - - - - A T R K I A I N T N	317
MFT2	266	A G S I L V K E W V F Q - - - - - A T K K V A I Q T N	287
ZnT-2	184	H G H S H G - - H S H - - - - - E D S S Q Q Q Q N	201
ZnT-3	203	P P H S H G - - S R G - - - - - A E Y A P L E E G	220
C.elegans	203	H S H S H G G G S S H - - - - - G H S H G G G N G	222
E.Coli	147	E E K N L N -	152
CZCD	142	Q S S S L N -	147
COT1	140	H G H G H G H S H G G I F A D H E M H M P S S H T H T H A H V D G	172
ZRC1	141	H S H S H G S V E S G - - - N N D L D I E S N A T H S H S H A S L	170
MFT1	318	S N -	319
MFT2	288	S N -	289
ZnT-2	202	P -	202
ZnT-3	221	P E Q P L P L G -	228
C.elegans	223	D -	223
E.Coli	153	- -	152
CZCD	148	- -	147
COT1	173	I E N T T P M D S T D N I S E I M P N A I V D S F M N E N T R L L	205
ZRC1	171	P N D N L A I D - E D A I S S P G P S G Q I G E V L P Q S V V N R	202
MFT1	320	- -	319
MFT2	290	- -	289
ZnT-2	203	- -	202
ZnT-3	229	- -	229
C.elegans	224	- -	224
E.Coli	153	- -	152
CZCD	148	- -	147
COT1	206	T P E N A S K T P S Y S T S S H T I A S G E N Y T E H N K R K R S	238
ZRC1	203	L S N E S Q P L L N H D D H D H S H E S - - - - - K K P G H R S	229
MFT1	320	- V L M A N A W H H R V D S L T S L V A L V A I S T G Y L V N - -	349
MFT2	290	- V L M A N A W H H R V D S L T S L V A L V A I T S S Y F F N - -	319
ZnT-2	203	- S V R A A F I H V V G D L L Q S V G V L V A A Y I I Y F K P - -	232
ZnT-3	230	T S V R A A F V H V L G D L L Q S F G V L A A S I L I Y F K P - -	260
C.elegans	225	I N V R A A F I H V L G D L L Q S L G V L V A A L F I Y F Q P - -	255
E.Coli	153	- - V R A A A L H V L G D L L G S V G A I I A A L I I Y F T - -	180
CZCD	148	- - V K G A Y L E V W S D L L G S V G V I A G A I I I R F T - -	175
COT1	239	L N M H G V F L H V L G D A L G N I G V M L S A F F I W K T D Y S	271
ZRC1	230	L N M H G V F L H V L G D A L G N I G V I A A A L F I W K T E Y S	262
MFT1	350	- I Q S L D T I G G L I V S G L I I K A G G E G M C I A I K E L I	381
MFT2	320	- T Q S L D N L G G L V V S G L I I K T G G Q G I L S S L K E L V	351
ZnT-2	233	E Y K Y V D P I C T F L F S I L V L G T T L T I L R D V I L V L M	265
ZnT-3	261	Q Y K A A D P I S T F L F S I C A L G S T A P T L R D V L R I L M	293
C.elegans	256	S W V I I D P I C T L V F S V I V L C T T I Y I L R D A M I V L L	288
E.Coli	181	G W T P A D P I L S I L V S L L V L R S A W R L L K D S V N E L L	213
CZCD	176	G W A W V D S A I A V L I G L W V L P R T W I L - - - - - - - -	199
COT1	272	W K Y Y T D P L V S L I I T G I I F S S A L P L S C K A S K I L L	304
ZRC1	263	W R Y Y S D P I V S L I I T I I I F S S A L P L S R R A S R I L L	295

FIG. 3—continued

Myc-epitope marked YMR177W carried on a centromeric plasmid did not reveal any specific band. A similar analysis, however, on cells transformed with a multicopy plasmid containing the Myc-tagged gene showed a specific band migrating at a molecular mass of 56 kDa on SDS-polyacrylamide gel electrophoresis. When the presence of the Myc-epitope is taken into consideration, the observed molecular mass is the same as that

predicted from the deduced sequence. Treatment of samples with endoglycosidase H had no effect on size of the protein, indicating that the protein does not contain any N-linked carbohydrate.

The Myc-tagged YMR177W was quantitatively recovered in a 10,000 × g membrane pellet, as expected from the presence of hydrophobic domains in the deduced sequence. To further de-

MFT1	382	DQSVSRD	DP	RYLEIETLVKDTLNKL	ISNNNSQK	414																																
MFT2	352	DQSIPTD	PP	RYLEIESVIKDSIGSL	KTDLDLQK	384																																
ZnT-2	266	EG	- - - -	TPKGVDFTTVKN	-LLLSVDGVEA-LH	291																																
ZnT-3	294	EG	- - - -	TPRNVGFEFVRD	-TLLSVPGVRA-TH	319																																
C.elegans	289	EG	- - - -	RPSNIDFAKVFSS	-SLEDIEGVKK-VH	314																																
E.Coli	214	EG	- - - -	APVSLDIAELKRRMCREI	PEVRN-VH	240																																
CZCD	0	-	- - - -	-	-	199																																
COT1	305	QA	- - - -	TPSTLSGDQVEG	-DLLKIPGIIA-IH	330																																
ZRC1	296	QA	- - - -	TPSTISADQIQR	-EILAVPGVIA-VH	321																																
MFT1	415	PYGLKE	LT	LLSSGPNLRG	H	LTLEVP	LQKWN	I	L	447																												
MFT2	385	SLHVRD	LT	ILASGPNLRATT	T	TLEVP	VLHSGQE	V	-	417																												
ZnT-2	292	SLH	I	WAL	T	V	AQ	- -	PVLSV	H	I	A	I	AQ	N	V	D	A	Q	-	AVL	321																
ZnT-3	320	ELHLW	A	L	T	L	T	-	-	H	V	A	S	A	H	L	A	I	D	S	T	A	P	E	-	AVL	349											
C.elegans	315	DLR	I	W	S	L	T	M	D	K	- -	I	A	L	S	V	H	L	E	I	D	A	N	S	Q	S	-	S	I	L	344							
E.Coli	241	HVH	V	W	-	M	V	G	E	K	- -	P	V	M	T	L	H	V	Q	V	I	P	P	H	D	H	-	A	L	L	269							
CZCD	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	199							
COT1	331	D	F	R	V	M	N	L	T	E	S	I	- -	F	I	A	S	L	H	I	Q	L	D	I	S	P	E	Q	F	-	T	D	L	360				
ZRC1	322	D	F	H	V	M	N	L	T	E	S	I	- -	Y	I	A	S	I	H	V	Q	I	D	C	A	P	D	K	F	-	M	S	S	351				
MFT1	448	GVNEFE	I	V	T	H	L	R	N	V	L	T	N	E	V	S	N	L	R	R	L	D	I	E	Y	V	E	E	-	-	-	-	480					
MFT2	418	G	I	R	F	L	E	N	A	I	S	T	I	R	E	D	L	R	M	K	V	P	N	V	G	K	V	D	V	E	F	V	D	V	450			
ZnT-2	322	K	V	A	R	D	R	L	Q	G	K	F	N	F	H	T	M	T	I	Q	I	E	S	Y	S	E	D	M	K	S	C	Q	E	C	354			
ZnT-3	350	A	E	A	S	R	L	Y	S	R	F	G	F	S	S	C	T	L	Q	V	E	Q	Y	Q	P	E	M	A	Q	L	R	C	382					
C.elegans	345	R	T	R	K	M	L	K	Q	T	Y	N	V	H	E	I	T	I	Q	I	E	E	F	G	A	N	R	S	D	C	G	K	377					
E.Coli	270	D	Q	I	Q	H	Y	L	M	D	H	Y	Q	I	E	H	A	T	I	Q	M	E	Y	Q	P	C	H	R	S	D	C	H	L	N	302			
CZCD	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	199			
COT1	361	A	K	I	V	R	S	K	L	H	R	Y	G	I	H	S	A	T	L	Q	P	E	F	I	T	R	E	V	T	S	T	E	R	-	392			
ZRC1	352	A	K	L	I	R	K	I	F	H	Q	H	G	I	H	S	A	T	V	Q	P	E	F	V	S	G	D	V	N	E	D	I	R	R	-	384		
MFT1	481	KN	- - - -	G	- - - - - - - - - -	E	E	N	E	H	I	K	G	Q	Q	N	Y	K	E	D	V	L	500															
MFT2	451	T	S	D	S	-	K	G	D	- - - - -	L	E	H	S	H	D	T	K	S	T	N	H	T	H	S	D	475											
ZnT-2	355	- - - -	Q	G	- - - - -	- - - - -	P	S	E	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	359			
ZnT-3	383	- - - -	Q	E	- - - - -	- - - - -	P	P	Q	A	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	388			
C.elegans	378	- - - -	D	F	- - - - -	- - - - -	P	T	K	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	382			
E.Coli	303	- - - -	E	G	- - - - -	- - - - -	V	S	G	H	S	H	H	H	H	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	313		
CZCD	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	199		
COT1	393	- - - -	A	G	- - - - -	- - - - -	D	S	Q	G	D	H	L	Q	N	D	P	L	S	L	R	P	410															
ZRC1	385	R	F	S	I	A	G	G	S	P	S	S	S	Q	E	A	F	D	S	H	G	N	T	E	H	G	R	-	K	K	R	S	P	416				
MFT1	501	I	K	H	D	H	T	N	T	H	I	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	510			
MFT2	476	S	A	D	T	H	T	H	K	H	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	484			
ZnT-2	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	359		
ZnT-3	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	388		
C.elegans	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	382		
E.Coli	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	313		
CZCD	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	199		
COT1	411	K	T	Y	G	T	G	I	S	G	S	T	C	L	V	D	D	A	A	N	C	N	T	A	D	C	L	E	D	H	-	-	-	-	439			
ZRC1	417	T	A	Y	G	A	T	A	S	S	N	C	I	V	D	D	A	V	N	C	N	T	S	N	C	L	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	442

FIG. 3—continued

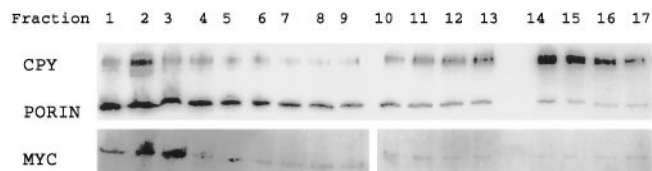


FIG. 4. Western blot analysis of subcellular fractions from Percoll gradients. Cells transformed with Myc-tagged YMR177W (*MFT1*) were homogenized and the homogenate was applied to a Percoll gradient. Fractions from the gradient were probed with antibodies to mitochondrial porin, vacuolar Cyp, or Myc. Fractions enriched in the epitope marked YMR177W were enriched for the porin and selectively depleted for Cyp.

fine the location of the protein a low speed supernatant (800 × g) from homogenized cells was applied to a Percoll density gradient. Fractions were collected and applied to SDS-polyacrylamide gel electrophoresis, and Western blots were probed using specific antibodies to defined membrane proteins. Most of the epitope-marked protein was found in the same fractions that were enriched for mitochondria as defined by the presence of the outer mitochondrial membrane protein porin. These fractions were depleted of endoplasmic reticulum (Erg25p), plasma membrane (Fet3p), and vacuoles (Cyp) (Fig. 4). To further confirm the location of YMR177W, cells transformed with a

high copy plasmid encoding a GFP-tagged fusion protein was examined by fluorescence microscopy. The protein is localized to mitochondria as its distribution is identical to that of the mitochondrial protein porin (Fig. 5). Similar results were obtained using cells transformed with YMR177W containing a carboxyl-terminal Myc-tag, in which the localization was examined by indirect immunofluorescence (data not shown). A YPL224C fusion protein containing a carboxyl-terminal Myc-tag was also localized to the mitochondria (Fig. 6). Based on their localization to mitochondria and their homology to transition metal transporters, we have termed these genes *Mitochondrial Iron (Fe) Transporter* in which YMR177W is *MFT1* and YPL224C is *MFT2*.

Evidence that MFT1 and MFT2 Affect Cytosolic Iron Concentration—Both *MFT1* and *MFT2* were identified due to their ability to suppress the poor growth of *fet6-2* in low iron medium. We considered that they could exert this effect by either being involved in the direct insertion of iron into this oxo-diiron protein, or by increasing the cytosolic concentration of iron and, thereby, indirectly providing more iron to the defective methyl sterol oxidase. Since these gene products are localized to mitochondria, we hypothesize that their effect on *fet6-2* is indirect and results from an increase in cytosolic iron concentration.

Several experiments indicate that the latter hypothesis is

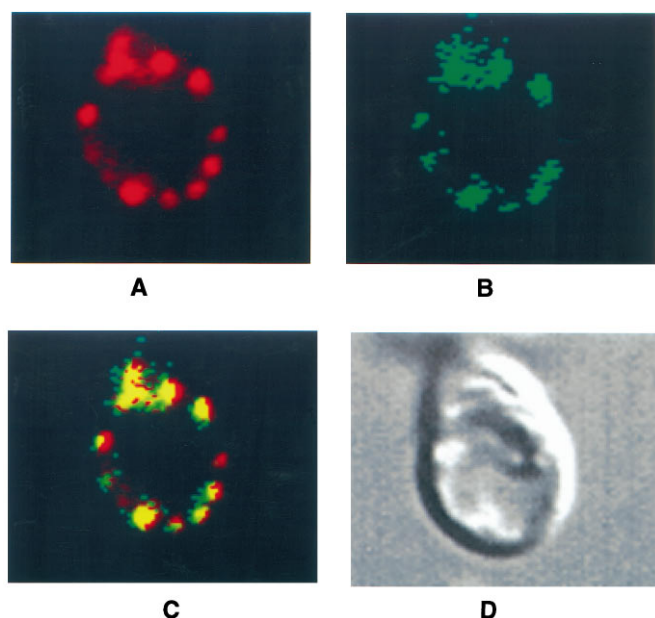


FIG. 5. Fluorescent localization of YMR177W (*MFT1*)- green fluorescent protein to mitochondria. Wild type cells (DY150) were transformed with a multicopy plasmid containing a fusion construct of YMR177W with a carboxyl-tagged GFP. Cells transformed with the plasmid were stained with antibodies to porin as described under "Materials and Methods." The same population of cells was examined for GFP fluorescence (green) (A); porin fluorescence (Texas Red) (B); a merge of A and B (C); and by Normarski optics (D).

correct. Cells containing a deletion in either *MFT1* or *MFT2* show no growth defect on either low or high iron medium. Cells with both genes deleted show a growth defect on low iron-medium. Growth on low iron-medium is absolutely dependent on the *FET3/FTR1* oxidase/permease high affinity iron transport system (1, 2). Measurement of the activity of this transport system in both wild type, deletion strains, and overexpressors, revealed no alteration in the rate of iron transport (data not shown). This result suggests that the effect of *MFT1* and *MFT2* are not exerted at the level of plasma membrane transport but at intracellular iron metabolism. To confirm this conclusion, wild type, deletion and overexpressor strains were grown in iron-free media, making cell growth solely dependent on intracellular iron stores. In this medium, the double deletion strain ($\Delta MFT1, \Delta MFT2$) grew to a lower saturation density than wild type cells, which did not grow as well as cells overexpressing *MFT1* (Fig. 7) or *MFT2* (data not shown). This result suggests that overexpression of *MFT1*p increases cytosolic iron by accessing internal pools.

Previously, we used the iron dependent toxicity of SNG to select cells that had defects in iron transport (7). SNG is an aminoquinone antibiotic that diffuses into cells and is reduced. Reduced SNG in the presence of ferrous iron and oxygen generates toxic hydroxyl radicals. Thus, cell viability in the presence of SNG can be used as an assay for cytosolic iron concentration. When grown on low iron medium, $\Delta MFT1, \Delta MFT2$ cells were more resistant to SNG than wild type cells, which were more resistant than cells overexpressing *MFT1* (Fig. 8). There was no difference in SNG sensitivity when cells were grown on YPD, a moderate to high iron containing medium (data not shown). These results indicate that overexpression of *MFT* genes can affect intracellular iron concentration. Incubation of either overexpressors or the double deletion strain in media containing either increased or decreased levels of copper, manganese, zinc, or cobalt, did not show any growth effect different from wild type cells. This result suggests that the *MFT* genes may be specific for iron transport.

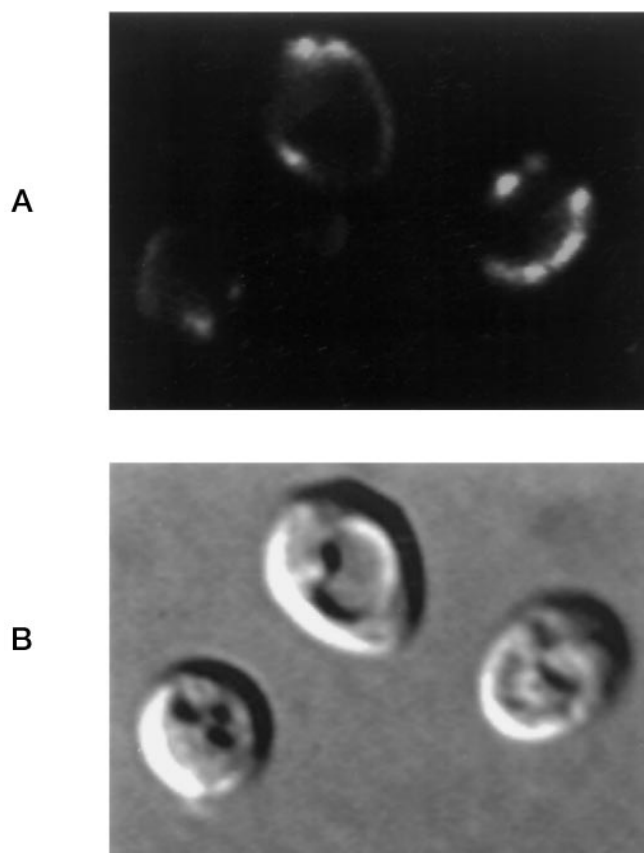


FIG. 6. Immunofluorescent localization of Myc tagged YPL224C (*MFT2*) to mitochondria. Cells transformed with a YEP plasmid containing a carboxyl tagged Myc-YPL224c gene were prepared for immunofluorescence as described under "Materials and Methods." A, immunofluorescence; B, Normarski.

Evidence That MFT1 and MFT2 Affect Mitochondrial Iron Accumulation—Many of the members of the extended gene family to which *MFT1/MFT2* belongs were identified as metal resistance genes. Expression of these genes confer resistance to the specific transition metals by either transporting them out of cells or into the lumen of intracellular vesicles. Consequently, the action of these genes permits cells to grow in supernormal levels of transition metals. To determine if *MFT1* or *MFT2* functioned in a similar manner, cells were incubated in supernormal levels of iron, and both whole cell and mitochondrial iron concentration determined by atomic absorption spectroscopy (Table I). Cells that overexpressed either gene showed a higher accumulation of iron than wild type cells. Further, this increased iron could be quantitatively accounted for by an increase in mitochondrial iron. Incubation of cells in increased concentrations of either cobalt, zinc, or manganese did not result in either increased cellular or mitochondrial levels of these transition metals.

DISCUSSION

We identified two different genes, *MFT1* and *MFT2*, which when overexpressed were capable of partially overcoming the growth defect attributed to a mutation in an endoplasmic reticulum iron-binding enzyme, methyl sterol oxidase. We believe that the iron binding site of Erg25p is in the cytosol and hence is sensitive to cytosolic iron. Overexpression of these two gene products in cells grown in low iron medium did not increase plasma membrane iron transport. Rather, the data suggest that overexpression increased cytosolic iron as a result of mobilization of iron from mitochondrial pools. In the absence of any exogenous iron, cells transformed with multicopy plasmids

containing either *MFT1* or *MFT2* grew longer than control cells, and were more susceptible to the iron mediated toxicity of SNG. Conversely, cells in which both genes were deleted were more SNG resistant than control cells and showed a lowered rate and degree of growth in iron free media. The fact that cells grew longer in iron free media supports the view that the effect of these genes is not on plasma membrane iron transport but on redistribution of iron from intracellular compartments.

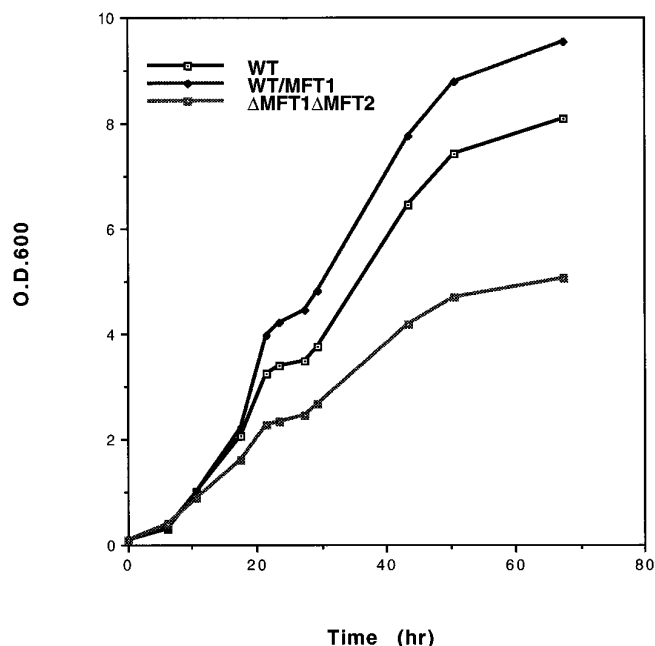
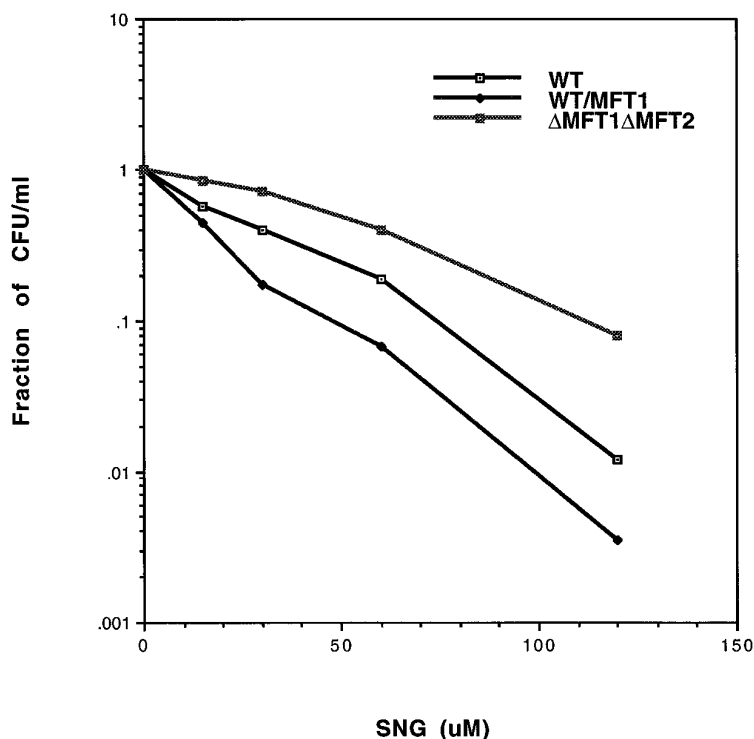


FIG. 7. Growth of cells that overexpress *MFT1* or have a deletion in *MFT1* and *MFT2* in iron-free-medium. Wild type cells or cells carrying either a multicopy plasmid expressing *MFT1*, or a strain with a double deletion ($\Delta MFT1$, $\Delta MFT2$) were grown in YPD. The cells were washed and resuspended in media made iron-deficient by the addition of BPS to YPD medium (BPSO). At the specified times, cell multiplication was assayed by measuring the optical density of the cultures using a wave length of 600 nm.

FIG. 8. SNG sensitivity of cells that either overexpress *MFT1* or have a deletion in the *MFT1/MFT2* genes. Wild type cells or cells carrying either a multicopy plasmid expressing *MFT1*, or a strain with a double deletion ($\Delta MFT1$, $\Delta MFT2$) grown in YPD were transferred to iron-limited medium (YPD BPS5) for 24 h. The cells were washed and exposed to the specified concentrations of SNG in iron-free medium for 2 h. The cells were then plated on to YPD medium and the number of colonies determined.



Based on subcellular fractionation and immunofluorescence both *MFT1* and *MFT2* encode mitochondrial membrane proteins. The major caveat to these studies is that overexpression is required to detect the tagged proteins and that overexpression may lead to mislocalization. In this instance that concern is lessened for two reasons. First, the protein is exclusively localized to a single organelle. Second, to our knowledge there is no evidence from published studies that overexpressed mitochondrial proteins are missorted to other organelles.

The *MFT* genes show amino acid homology to a gene family that encode transition metal transporters. All family members have six potential transmembrane domains and are not members of the ABC family. While a number of these proteins are simply anonymous open reading frames, many have definable functions or are associated with phenotypes. For example *COT1*, a yeast gene that encodes a mitochondrial protein, was identified through a screen that selected for genes that when overexpressed conferred resistance to increased Co (17). The yeast *ZRC1* gene that confers resistance to cadmium and zinc (21) is also a member of this family. Similarly, *czcd* was identified as a gene on a plasmid that conferred resistance to cadmium, zinc, and cobalt in the bacteria *Alcaligenes eutrophus* (20). This gene has homology to genes found in *E. coli* and other

TABLE I

MFT1 and *MFT2* affect mitochondrial iron accumulation

Cells were grown to OD 3.0 in CM-Ura/glycerol/ethanol medium with 250 μM FeSO_4 . For whole cell iron measurement, cells were washed twice in ice-cold 50 mM Tris-HCl, pH 6.5, 10 mM EDTA, washed once in water, and analyzed for iron content through atomic absorption spectrophotometer. For mitochondrial iron, subcellular fractionation was performed as described under "Materials and Methods." Purified mitochondrial proteins were washed and dissolved in lysis buffer (25 mM Tris, 150 mM NaCl, pH 7.4). Protein concentrations were measured by BCA.

Strain ^a	Whole cell iron	Mitochondrial iron
	nmol Fe/mg protein	
WT/vector	2.04 \pm 0.08	5.38 \pm 1.52
WT/MFT1	5.41 \pm 0.04	33.99 \pm 2.53
WT/MFT2	5.35 \pm 0.09	25.43 \pm 0.50

^a WT, wild type.

bacteria, and with lower degrees of homology, to genes in the Archea. Other homologous genes include endosomal or synapto-somal zinc transporters present in mammalian cells (18, 19). Based on their location, as well as, direct physiological studies it is suggested that these proteins may be H⁺/metal co-transporters (20). Proteins located in either the bacterial membrane (Czcd) or mitochondrial membrane (Cot1p, MFT1p, MFT2p) may also utilize a pH gradient for transport activity (22). The proteins encoded by the mammalian zinc transporters, ZNT2 and ZNT3, are located in subcellular organelles which also may be acidic (18, 19). Each one of these proteins contains a histidine-rich sequence. The exact sequence is different for each of the family members, although the histidine rich region in *MFT1* and *MFT2* are extremely similar to each other. The role of this sequence is unknown. Two likely hypotheses are that this sequence is required to bind metals prior to transport or that it is a metal binding domain responsible for regulating the activity of these transporters.

That the *MFT* genes act as "metal resistance" genes is suggested by the observation that when cells expressing these genes were placed in high iron medium iron was accumulated above that seen in control cells. The ability to accumulate metal is also seen in yeast overexpressing the *COT1* gene that mediates cobalt resistance (17). In cells that overexpress *MFT1* or *MFT2*, the increased cellular iron is located within the mitochondria. The fact that the double deletion strain can grow well on both glycolytic and respiratory substrates indicates that under normal physiological conditions these genes are not responsible for the delivery of iron to either ferrochelatase or mitochondrial iron binding proteins.

The action of the *MFT* genes allows cells to utilize iron when cells grown in high iron are placed in iron-restricted medium. Based on this observation, we surmise that mitochondrial iron can be utilized for purposes other than heme biosynthesis. This line of reasoning suggests that mitochondria can act as a reservoir for iron. Iron, particularly in the presence of reactive oxygen intermediates, can be toxic. Indeed, the mitochondria is the major source of reactive oxygen intermediates. Cells overexpressing *MFT1* or *MFT2* are perfectly capable of growth on respiratory substrates, suggesting that the stored iron is not toxic. Conditions exist, however, in which mitochondrial iron accumulation can lead to toxicity. This has recently been demonstrated in cells bearing a deletion in the *YFH1* gene, a gene that is homologous to the mammalian gene Frataxin (23). Defects in Frataxin are responsible for Friedreich's ataxia, a lethal disease. Cells with a defective *YFH1* gene also accumulate iron in mitochondria. Such cells show a respiratory defect and

an increased sensitivity to H₂O₂. The observation that cells with overexpressed *MFT* genes in high iron conditions also accumulate mitochondrial iron suggest that iron accumulation *per se* need not be toxic. These observations suggest that strains overexpressing *MFT1/MFT2* or carrying a deletion in *YFH1* may accumulate iron into different mitochondrial sub-compartments or that *YFH1* may affect the conditions in which mitochondrial iron is stored. Additionally, the amount of iron found in mitochondria in $\Delta yfh1$ (10 \times) is much greater than in *MFT1/MFT2* overexpressors (2–5 \times). This may suggest that toxicity is defined by the absolute magnitude of mitochondrial iron accumulation. Experiments are in progress to distinguish between these hypotheses.

Note Added in Proof—The family of genes described here fit the characteristics of the cation facilitator family described by Paulsen and Saier (Paulsen, I. T., and Saier, M. H. (1997) *J. Membr. Biol.* **156**, 99–103).

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